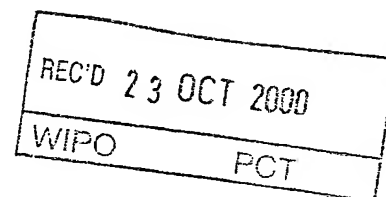


Helsinki 9.10.2000



ETUOIKEUSTODISTUS
PRIORITY DOCUMENT



Hakija
Applicant

Valtion teknillinen tutkimuskeskus
Espoo

Patenttihakemus nro
Patent application no

19991781

Tekemispäivä
Filing date

20.08.1999

Kansainvälinen luokka
International class

C12N

Keksinnön nimitys
Title of invention

"Improved biotechnical production method"
(Parannettu biotekninen tuotantomenetelmä)

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IMPROVED BIOTECHNICAL PRODUCTION METHOD

Background of the invention

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This invention relates to a novel method for decreasing the foam formation during the cultivation according to the preamble of claim 1, to a product produced by the method according to the preamble of claim 12 and to a novel production host strain with decreased foam formation during cultivation according to the preamble of claim 15.

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When microorganisms are cultured in liquid growth medium in laboratory vessels or in small or large scale bioreactors, a common feature is foaming. In particular, this is a problem in aerated bioreactor or, as they are usually called, fermentor cultivations. Biochemically, the term "fermentation" refers to the process of ethanol production in yeasts by anaerobic metabolism. Modern aseptic submerged "fermentation" of individual selected microbes is used for production of cell mass, proteins such as enzymes and antibodies, and other metabolites such as antibiotics, amino acids and organic acids. The main microorganisms used in industry in fermentation are fungi, especially filamentous fungi and yeast, especially filamentous fungi and yeast, and bacteria, such as *Bacillus* spp., *Escherichia coli* and *Streptomyces* spp.

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Typically only 70-80% of the fermentation vessel volume is filled with liquid and a gas space occupies the top portion of the tank. The combined action of aeration and agitation of the liquid promotes the formation of a foam on the liquid surface and normally all aerated fermentation broths foam, which is why a large gas space is required. Foam impedes gas mass transfer from the broth to the head space, forcing foam out of the vessel and contaminating the system when collapsed foam re-enters the fermentor. This means that some foam control method should always be included in fermentations. The two methods most commonly used for commercial fermentations are mechanical foam breakers and/or addition of antifoam agents.

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A mechanical foam breaker is a high-speed impeller designed very much like a centrifugal pulp impeller. Foam is drawn into the impeller, where it is collapsed by strong mechanical forces. This is not suitable for delicate organisms. The impeller must be mounted on its own

shaft and driven independently of the main agitator. This requires a separate agitator seal, which is a potential source of contamination and other problems. Mechanical foam breakers also represent a considerable capital outlay.

- 5 Chemical antifoam agents collapse foams by altering their surface tension characteristics. Sterile antifoam agent is usually pumped into the fermentor automatically from an addition vessel. Addition of antifoam agent results in a possible contamination risk because the polymeric water-free liquid agents are difficult to sterilize. The choice of antifoam agent cannot be made only on the basis of its compatibility with the fermentation, but also with
- 10 product recovery, concentration and purification in downstream processing. For example particularly silicon-based antifoam agents may decrease dramatically the permeate flux through certain types of membrane filters even at very low concentrations. Hydrophobic antifoam agents bind to the hydrophobic ultrafiltration membranes, lowering the permeate flux, and may change dramatically the apparent molecular weight cut-off of the membranes.

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The first antifoam agents to be used in fermentation were silicone-based fluids. More recently, oil-based antifoam agents with a chemical structure consisting of polymers of e.g. ethylene and propylene oxides with esters of long-chain fatty acids have commonly been used. All antifoam agents have a "cloud point", above which they are essentially insoluble in

20 water. The functional properties of the antifoam agents operate at temperatures above the cloud point. Therefore the cloud point of the antifoam agent should be below the temperature of the fermentation. However, for example membrane filtration should be operated at temperatures below the cloud point, so that the antifoam agent will be water-soluble and therefore have minimal effect on the filtration procedure. Antifoam agents are available with

25 very variable cloud points.

Most antifoam agents, because of their hydrophobic nature, are difficult to sterilise. They may also represent a considerable cost outlay. A good antifoam control system should also include an option to reduce automatically the air flow and the agitation speed when foaming

30 overwhelms the system and the fermenter is in danger of being emptied. This is necessary to avoid damage to the drive system if the fermenter is emptied because of overfoaming.

Because of their function of disrupting air-filled bubbles both above and within the fermentation broth, antifoam agents considerably reduce the oxygen transfer rate. By contrast, detergents usually enhance oxygen transfer rates. When antifoam agents and detergents are present simultaneously, they exert compensating effects. Oxygen transfer rates depressed owing to the addition of antifoam agent result in decreased dissolved oxygen levels at a constant rate of agitation and aeration. Antifoam agents decrease the surface tension, resulting in reduced volumetric mass transfer coefficient (k_La) values. This leads to a need for increased aeration and agitation, which in turn enhances foam formation and necessitates further addition of antifoam agent which further enhances the problems created by antifoam agent. The positive feedback effects of foaming and agitation/aeration represent a major problem in many commercial fermentation processes. A further practical disadvantage is reduced sensitivity and accelerated ageing of probes due to clogging by the polymer components of the antifoam agents.

Production media used in industrial fermentations often contain insoluble polymers. The presence of these insoluble medium components considerably aggravates the problem of foaming during fermentation. The foam produced during cultivation combines with unutilised solid particles from the medium to form a composite foam with strong physical properties and undesirably high persistence. This foam-solids conglomerate can remain attached to the sides of the vessel and to other steel structures in the fermenter headspace independently of support from below, and contact between the broth and foam layers is disestablished. Addition of antifoam agent to the fermentation is therefore not successful in degrading the secondary foam structure. Thus after establishment of contact between the upper foam layer and the tip of the antifoam probe, the subsequent automatic addition of antifoam agent does not disrupt the foam layer. In this case, addition of antifoam agent may, and frequently does, continue indefinitely until the addition vessel becomes empty. Despite continuing addition of antifoam agent, the conglomerate foam layer continues to rise in the fermenter headspace, eventually entering the exhaust line and blocking the outlet filter. This in turn prevents the passage of air through the broth and the dissolved oxygen decreases to zero, with disastrous effects on the production process. This whole cycle may occur in a short period of only 1-2 hours.

Oxygen transfer rate is affected by the microbial species, its morphology and concentration. Species with more complex morphology (pellets versus filaments, pellets do not have

significantly higher oxygen demand and uptake but clearly higher maximum oxygen transfer rate) lead to lower oxygen transfer rates. Increased viscosity due to the high mycelial concentrations (pseudoplastic moulds) concentration leads to reduced oxygen transfer rates.

- 5 Mycelial morphology also affects the process productivity and kinetics. In some cases the small pellets are optimal for the production of desired product whereas in other cases filamentous growth has been found to be optimal. The morphology also affects the downstream processing and filtration properties of the culture liquid as larger particles are easier to separate e.g. in vacuum drum filtration. Growth in the form of pellets usually leads
10 to an interstitial culture fluid with greater clarity and lower viscosity than in the case of diffuse mycelial growth.

Because of the several problems caused by foam formation during fermentation there is a high demand for new ways to hinder or cut down the foam formation.

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SUMMARY

It is an aim of the present invention to eliminate the problems associated with the prior art and to provide a solution to the problems caused by foaming during cultivation of various kinds of
20 microorganisms. The present invention provides a totally new way to solve the foaming problem, which results in minimal or no need to use antifoam agents.

25 The present invention provides in particular a method to eliminate or diminish the production of proteins, polypeptides or peptides associated with foam formation during cultivation of microorganisms. The method comprises genetic modification of desired microorganisms not to produce proteins, polypeptides or peptides associated with foam formation, or to produce them only in essentially reduced amounts. Such proteins, polypeptides or peptides are recoverable from foam produced in cultivation of the microorganisms. The microorganisms are selected from the group comprising yeast, fungi, bacteria, plant cells and animal cells.
30 Preferably they are production strains used in producing desired proteins, polypeptides, metabolites or biomass. Typically such strains are genetically modified to produce these products in an efficient way.

One object of the present invention is a method for decreasing the level of foam formation during cultivation of a microorganism, comprising the steps of

- modifying the microorganism in such a way that the microorganism does not produce an essential amount of proteins, polypeptides or peptides associated with foam formation during
- 5 cultivation of the microorganism; and
- cultivating the microorganism under suitable culture conditions.

More specifically, the method is mainly characterized by what is stated in the characterizing part of claim 1.

10

The "modification" of the microorganism is preferably "genetic modification", which means that at least one of the DNA sequences, or parts of it, encoding proteins, polypeptides or peptides associated with foam formation is modified not to be expressed and/or secreted. The genetic modification comprises various methods directed to the regulatory region of the DNA

15 sequence encoding the desired protein, polypeptide or peptide or various methods with which the DNA sequence can be inactivated, such as mutagenesis or deletion, or the genetic modification comprises various methods directed to DNA sequences encoding proteins regulating the production of proteins, polypeptides or peptides associated with foam formation. The genetic modification according to this invention is preferably made by

20 inactivating the desired DNA sequence or sequences of the proteins, polypeptides or peptides associated with foam formation. More preferably, the genetic modification is made by deleting the desired DNA sequence or sequences. This is because deletion is the most powerful technique to diminish the effect of the proteins, polypeptides or peptides associated with foam formation.

25

Molecules associated with foam formation during cultivation are various proteins, polypeptides or peptides recoverable from the foam formed in cultivation of the microorganism or their regulatory proteins. Such molecules comprise proteins, polypeptides or peptides, which regulate the production of foam-forming proteins, polypeptides or peptides

30 which are responsible for foam formation. Preferably they are proteins, polypeptides or peptides, which are foam-forming i.e., which are responsible for foam formation. Such proteins, polypeptides and peptides comprise hydrophobic or amphipathic proteins, polypeptides or peptides, hydrophobins, or amphipathic surface active molecules, or proteins,

polypeptides or peptides associated with lipids. Preferably the proteins, polypeptides or peptides associated with foam formation are hydrophobins. Hydrophobins are among the most abundantly produced proteins of fungi. All fungi studied hitherto for the presence of hydrophobins have been shown to produce one or more of them.

5

Because many microbial production hosts are known to produce foam-forming proteins, polypeptides or peptides during cultivation, the present invention can be applied to solve the foaming problem in the cultivation of various different kinds of microorganisms.

- 10 According to a one preferred embodiment of this invention the microorganism is a fungus. More preferably, the fungus is *Trichoderma*.

According to a preferred embodiment of this invention the proteins, polypeptides or peptides, the production of which is eliminated or diminished, are hydrophobins from *Trichoderma*.

15

According to a highly preferred embodiment of this invention the hydrophobins are hydrophobin I (HFBI) or hydrophobin II (HFBII). The invention comprises that a *Trichoderma* strain is genetically modified not to produce essential amounts of HFBI and/or HFBII. Preferably the DNA sequences encoding hydrophobin I (HFBI) or hydrophobin II

20 (HFBII) or both are inactivated in the *Trichoderma* strain.

According to another preferred embodiment of this invention the microorganism belongs to bacteria. The host strain may be any bacterial host producing proteins, polypeptides or peptides associated with foam formation during cultivation. Preferably the host strain is *E. coli*, or belongs to the genus *Bacillus* or *Streptomyces*.

25

Another object of the present invention is a new production host strain, which is genetically modified not to produce essential amounts of one or more of the proteins, polypeptides or peptides associated with foam formation when the non-modified production host strain is

30

cultivated.

More specifically, the production host strain is mainly characterized by what is stated in the characterizing part of claim 15.

One further object of the present invention is a product produced by the microorganism cultivated by the method of this invention as stated in the characterizing part of claim 12. The product can be any natural or recombinant protein, peptide, metabolite, antibiotic, fusion
 5 protein or even the cells themselves. The product is preferably a recombinant product.

The present invention results in various advantages. When little or essentially no foam is formed during the cultivation of a microorganism, no or only low levels of antifoaming agents are needed. This results in significantly easier downstream processing .

10

The cultivation of a microorganism modified according to this invention results in no or substantially reduced overfoaming. Hence, the yield is higher, when the product losses are smaller.

15 One clear advantage is also that the contamination risk is significantly lower when there is no need to add antifoam agents, which are difficult to sterilize and because the growth medium does not come into contact with the surface or other potentially nonaseptic areas of the bioreactor system.

20 According to this invention there will be less attachment of the cultivation medium to the fermentor surfaces, electrodes, impeller etc. Thus the distribution of cell mass in the fermentor is more homogenous. In addition the malfunction of electrodes is decreased, which in thurn improves the controllability of the fermentation.

25 As a result of this invention the overall productivity will be improved. One or several of the following parameters: product/protein secretion ratio, the specific productivity/protein ratio or productivity/fermentation ratio (more liquid in fermentor) will be higher compared to the ratios in cultivations wich are not modified according to this invention.

30 One further advantage is that the productivity will be better due to altered morphology. By inactivation of one or more hydrophobin genes it is possible to control the morphology of the strain inbetween filamentous or pellet-forming growth.

The invention is of advantage particularly when cultivating microorganisms on a culture medium comprising insoluble components. Antifoam agents cannot degrade efficiently the composite foam formed from proteinaceous foam produced by the microbe metabolism and unutilized solid particles. According to this invention no or only diminished amount of foam
5 is formed and hence no undegradable secondary structure foam is hampering the cultivation.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the *hfb1* genomic sequence (SEQ ID No 1) of 2868 bp, which contains protein
10 encoding (and introns) and flanking sequences (not all of the subcloned flanking sequences are sequenced). *hfb1* gene sequence is underlined. MunI restriction sites used for cloning of the marker gene are in bold and underlined.

Figure 2 shows *hfb2* genomic sequence (SEQ ID No 2) of 3585 bp, which contains protein
15 encoding (and introns) and flanking sequences. *hfb2* gene sequence is underlined. BglI and EcoRV restriction sites used for cloning of the marker gene are in bold and underlined. Polylinker sequence of the cloning vector is in Italics and the restriction sites within the polylinker used for cloning purposes are underlined.

Figure 3 depicts plasmid pTNS24

Figure 4 depicts plasmid pTNS 27

20 Figure 5 depicts plasmid pMQ113

Figure 6 depicts plasmid pTH1

Figure 7 depicts plasmid pTH2

Figure 8 depicts growth parameters of *Trichoderma* strains in a fermentor cultivation on
lactose (DO= dissolved oxygen).

25 Figure 9 depicts the production of soluble protein in a fermentor cultivation on cellulose medium.

DETAILED DESCRIPTION OF THE INVENTION

30 The term **cultivation** denotes here any methods used to grow microbial cells in a laboratory container or in large scale. The present invention is particularly useful to be applied to bioreactor cultivations, which are aerated and/or agitated.

The growth or cultivation medium should be chosen according to the microorganism in question. To achieve optimal growth of the microorganism and/or optimal production of the desired product, nutrients, aeration and pH conditions must be optimal for the microorganism. The cultivation medium may optionally be a medium which does not induce or which hinders the production of proteins or polypeptides, which are associated with foam formation during cultivation.

The term fermentation denotes here any bioreactor cultivations, preferably cultivations, in which agitation and/or aeration are used. The fermenter is preferably a normal fermentor, but the invention may be applied to airlift or other fermentor types.

The term **microorganism** denotes here bacteria, yeast, fungi, plant and animal cells. Preferably the invention is applied to fungi or bacteria, more preferably to fungi.

15 **Fungal host strains** of this invention comprise *Aspergillus spp.*, *Trichoderma spp.*, *Neurospora spp.*, *Fusarium spp.*, *Penicillium spp.*, *Humicola spp.*, *Tolypocladium geodes*, *Kluyveromyces spp.*, *Pichia spp.*, *Hansenula spp.*, *Candida spp.*, *Yarrowia spp.*, *Schizosaccharomyces pombe*, *Saccharomyces spp.*

20 **Bacterial host strains** of this invention comprise *Bacillus spp.*, *Zymomonas spp.* and *Actinomycetales*, such as *Streptomyces spp.*, *Nocardia spp.* and *Escherichia coli*

A **production host strain** denotes here any microorganism, which is selected or genetically modified to produce efficiently a desired product and is useful for industrial application. The host strain is preferably a recombinant strain modified by gene technological means to efficiently produce a product of interest.

The term **genetic modification** comprises recombinant DNA technology or gene technology, cell fusion and hybridisation, mutagenesis, induced polyploidy, conjugation, transduction, transformation and injection of heritable material into a cell.

Genetic modification of a microorganism not to produce an essential amount of at least one of the proteins or peptides associated with foam formation during cultivation of the non-

modified microorganism strain comprises that DNA sequences encoding at least one of the proteins, polypeptides or peptides associated with foam formation are modified by various genetical methods not to be expressed or secreted. Alternatively DNA sequences encoding regulatory proteins regulating the production of foam-forming proteins, polypeptides or peptides or the DNA sequences encoding foam-forming proteins, polypeptides or peptides are genetically modified. Genetic modification comprises furthermore that regulatory regions of the the genes encoding proteins, polypeptides or peptides associated with foam formation are genetically modified. According to a preferred embodiment of this invention the DNA sequences encoding foam-forming proteins, polypeptides or peptides are made inactive. The inactivation may be made by any suitable conventional or molecular biology method well known in the art. The modification is preferably made by recombinant DNA techniques, such as by site directed mutagenesis or deletion. Most preferably the inactivation is made by deleting the DNA sequence encoding the chosen protein, polypeptide or peptide.

Cultivation products may preferably include desired proteins, polypeptides, peptides, metabolites or cell mass.

The expression proteins, polypeptides or peptides associated with foam formation refers here to any molecules, which are associated with foam formation during cultivation of a microorganism and which are recoverable from the foam formed during cultivation of the microorganism. The group comprises foam-forming proteins, polypeptides or peptides and proteins, polypeptides or peptides regulating the production of foam-forming proteins or peptides. Preferably the group comprises hydrophobic or amphipathic proteins, polypeptides or peptides, hydrophobins or amphipathic surface active molecules and proteins, polypeptides or peptides associated or covalently linked to lipids. Such proteins or peptides are e.g. lipoaminoacids and lipopeptides, lipoproteins. Preferably the proteins or peptides associated with foam formation are hydrofobins.

Examples of molecules responsible for foam formation during cultivation are hydrophobic, amphipathic proteins and peptides. Hydrophobins are secreted amphipathic proteins with interesting physico-chemical properties that have recently been discovered from filamentous fungi (Wessels, 1994; Wösten and Wessels, 1997; Kershaw and Talbot, 1998). They were first discribed at DNA level (Schuren and Wessels, 1990) and later identified as proteins that are

active in the interaction between the fungal surface and the environment. Although the term "hydrophobin" had been used earlier to denote any hydrophobic material on the microbial surfaces, the term hydrophobin was used by Wessels to name these proteins (Wessels et al., 1991a,b). Hydrophobins are among the most abundantly produced proteins of fungi and all fungi studied hitherto have produced one or more hydrophobins.

One characteristic feature of these proteins is their moderate hydrophobicity. They are usually small proteins, approximately 70 to 160 amino acids, containing six to ten, usually eight cysteine residues in conserved pattern. The eight cysteine residues (Cys) have a conserved spacing: X_{2-38} - Cys - X_{5-9} - Cys - Cys - X_{11-39} - Cys - X_{8-23} - Cys - X_{5-9} - Cys - Cys - X_{6-18} - Cys - X_{2-13} , in which X means any other amino acid. However, multimodular proteins with one or several hydrophobin domains and e.g. proline-rich or asparagine/glycine repeats, or hydrophobins containing less than eight cysteine residues have also been characterized (Lora et al., 1994; Lora et al., 1995; Arntz and Tudzynski, 1997; de Vries et al. 1999).

Hydrophobins have been divided into two classes based on their hydropathy profiles and physico-chemical properties (Wessels, 1994). Class I hydrophobins form highly insoluble assemblages, whereas Class II hydrophobins assemblages are less stable and are soluble e.g. in 60% ethanol and 2% SDS. However most probably some hydrophobins exhibit characteristics between these two classes. Although more than 30 gene sequences for hydrophobins have been published (Wösten and Wessels, 1997), only few of the proteins have been isolated and studied. Today most protein data exists for the hydrophobins SC3 of *Schizophyllum commune* (Class I), cerato-ulmin of *Ophiostoma ulmi* and cryparin of *Cryphonectria parasitica* (Class II). Other isolated hydrophobins include at least SC4 of *S. commune*, ABH1 and ABH2 of *Agaricus bisporus* and EAS of *Neurospora crassa*.

The most characteristic feature of hydrophobins is that they self-assemble at hydrophilic/hydrophobic interfaces. By self assembly at the interfaces between the hydrophilic cell wall and a hydrophobic environment (air, oil, soil), emergent structures are covered with an amphipathic membrane. The transition of hydrophilic to hydrophobic cell surface allows formation of aerial hyphae, facilitates dispersion of spores by wind, maintains open air channels within fruiting bodies, and mediates hyphal attachment and signaling of surface hydrophobicity.

Hydrophobins make the areal hyphae water resistant and water repellant. Moreover they are involved in complex interhyphal interactions.

Hydrophobins do not only assemble at the cell wall/air or liquid interface but also at the interface between the hydrophilic cell wall and a hydrophobic solid. Hydrophobins firmly glue fungal hyphae and hydrophobic surfaces together (Wösten et al., 1994a). The adhesion of the hyphae to the solid surface is due to the amphipathic nature of the hydrophobin membrane, i.e. each side interacts with one of the two surfaces.

- Hydrophobins also assemble at liquid-air interfaces. Several hydrophobins such as SC3 of *S. commune*, COH1 of *Coprinus cinereus*, ABH3 of *A. bisporus* and POH2 and POH3 of *Pleurotus ostreatus* are secreted into the culture medium (Wösten et al., 1999). Filamentous fungal species, such as *Aspergillus nidulans*, *A. niger*, *A. oryzae*, *Neurospora crassa* and *Penicillium chrysogenum* produce amphipathic proteins, most probably hydrophobins to the culture medium (de Vries et al., 1993, Wessels, 1997).

Hydrophobins are secreted as monomers, but when they encounter an air-water interface or an interface with hydrophobic surface, they aggregate to a larger polymeric complex. This thin layer formed is hydrophobic on one side and hydrophilic on the other. The SC3 assemblages, as well as those of cerato-ulmin and cryparin (Wessels, 1997), form on gas-liquid or oil-liquid interphases thus stabilizing air bubbles or oil droplets in water. The self-assembly of purified SC3 hydrophobin into an amphipathic layer occurs also on hydrophilic and hydrophobic surfaces (Wösten et al., 1993; Wösten et al., 1994b). This film is very strongly attached to the surface and not broken, for instance, by hot detergent. The hydrophobic side of the layer on hydrophilic surfaces shows properties similar to those of teflon (Wessels, 1994). Upon shaking SC3 hydrophobin-containing solutions, the protein monomers form 10 nm rodlet-like aggregates. These structures are similar to those found on surfaces of fungal aerial structures.

Several biosurfactants that decrease water surface tension are known in the literature (Wösten and Wessels, 1997). Surface activity of proteins is generally low but hydrophobins belong to surface-active molecules, their surfactant capacity being at least similar to traditional biosurfactants such as glycolipids, lipopeptides/lipoproteins, phospholipids, neutral lipids and fatty acids (Wösten and Wessels, 1997). In fact SC3 hydrophobin is the most potent

biosurfactant known. It lowers the water surface tension to 24 mJm^2 at a concentration of $50 \text{ }\mu\text{g/ml}$ due to a conformational change during self-assembly of monomers into an amphipathic film (Wösten and Wessels, 1997). Similarly to SC3 hydrophobin also HFBI and HFBII hydrophobins of *T. reesei* have been shown to reduce water surface tension (our unpublished
 5 results). The decreased surface tension leads to more stable gas bubbles, thus hydrophobins and other amphibatic polypeptides stabilise foam.

Hydrophobin-like molecules vary in their properties. For example, rodlet-forming capacity has not been attributed to all hydrophobins (such as some class II), or they might have a weaker
 10 tendency to form stable aggregates (Russo *et al.*, 1982; Carpenter *et al.*, 1992). Another group of fungal amphipathic proteins are repellents (Kershaw and Talbot, 1998). Other type of proteins and polypeptides responsible for foam formation may consequently have only some of the features attributed to hydrophobins. Such examples are SapB and streptofactin, which are surface-active peptides secreted into the culture medium by *Streptomyces coelicor* and *S. tendae*,
 15 respectively (Willey *et al.*, 1991, Richter *et al.*, 1998).

Other proteins within the scope of protection of this invention are microbial amphipathic surface active molecules, the main classes of which are glycolipids, lipoaminoacids and lipopeptides, lipoproteins, lipopolysaccharides, phospholipids, mono- and diglycerides and fatty acids
 20 (Cameotra and Makkar, 1998; Lang and Wullbrandt, 1999). The molecular structures of these comprise a hydrophilic portion, which may consist of mono-, oligo or polysaccharides, amino acids or peptides or carboxylate or phosphate groups, and a hydrophobic portion, which is composed of saturated or unsaturated (hydroxy)fatty acids or fatty alcohols. Examples of this
 25 type of molecules are lipopeptides of *Bacillus licheniformis* and surfactin of *B. subtilis* (Wösten and Wessels, 1997). However in contrast to these surfactants, surface activity of hydrophobins is not dependent on a lipid molecule but apparently caused solely by the amino acid sequence. The glycan part of SC3 is mainly present in the hydrophilic part of the protein, probably contributing to the hydrophilicity of this part of the protein. However, most
 30 hydrophobins are not glycosylated and none of them is reported to be a lipoprotein. Furthermore, surface activity of hydrophobins seems to depend on a conformational change in the molecules during assembly rather than on a diffusion-limited adsorption to the interface (van der Wegt *et al.*, 1996, Wösten and Wessels, 1997).

Very little is known about the 2D and 3D structures of hydrophobins and the changes that occur upon self-assembly. However both hydrophobin classes are speculated to contain two domains which are stabilised by two disulfide bridges (Wösten and Wessels, 1997).

- 5 Strategies to find and inactivate hydrophobins and other hydrophobic/amphipathic proteins and polypeptides are well known in the art and include techniques such as screening of genomic or cDNA libraries with hybridization techniques using homologous/heterologous DNA fragments and oligonucleotides designed on the basis of conserved regions in the protein/peptide encoding region. The sequence diversity of hydrophobins means, however, 10 that isolation of hydrophobin-like genes on the basis of sequence homology may prove difficult. Few reports exist in which nucleic acid similarity has been exploited to isolate a hydrophobin gene using heterologous hybridization (e.g. Muñoz et al. 1997).

- However, several techniques exploiting purified protein for isolation of the corresponding 15 gene are known to a person skilled in the art. After proteins, polypeptides or peptides associated with foam formation have been purified, the corresponding genes are isolated using suitable techniques such as e.g. screening of expression libraries using antibodies raised against purified proteins, or PCR cloning or screening of genomic and/or cDNA libraries using oligonucleotides designed on the basis of N-terminal or internal protein sequences.

...20

- Proteins, polypeptides, or peptides associated with foam formation may be purified on the basis of their properties. They can be recovered from the foam formed during the cultivation of a strain or caused by bubbling gas through the medium. Foam-associated proteins, polypeptides and peptides may further be recovered from aggregates caused by freezing of 25 culture medium. Proteins, polypeptides or peptides, the prevention of whose production is useful according to the present invention, can also be obtained by applying the cells, cell extracts or culture media of a strain to aqueous two-phase system (ATPS) and recovering the proteins separated into the phase containing the hydrophobic phase material as shown for hydrophobins (Hyytiä et al. 1999).

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- WO 96/41882 suggests the production of hydrofobins from edible fungi for food industry. The publication describes the overexpression of hydrophobins, but does not suggest their inactivation.

According to this invention a microorganism strain is genetically modified not to produce an essential amount of at least one of the proteins or polypeptides or peptides associated with foam formation during the cultivation of the non-modified microorganism host. **An essential**
 5 **amount** means here that the host strain produces at least 50 % less amphipathic proteins, polypeptides or peptides, preferably 60- 80 %, most preferably 80 -100% less amphipathic proteins, polypeptides or peptides compared to the non-modified parent host strain.

Decreasing the level of foam formation during cultivation means here that the foam formation
 10 is lowered at least 30%, preferably 40- 80 %, most preferably 80-100% compared to the foam formation during cultivation of the non-modified parent host strain.

The decreased foam formation results in savings of antifoam agents and increase in fermentation working volume. The saving of antifoam agent is at least 30%, preferably 40-
 15 80%, most preferably 80-100%. The increase in fermentation working volume is at least 5%, preferably 10-20%.

A deletion vector denotes here a vector comprising a DNA sequence encoding a marker gene and so called flanking regions, which make it possible to delete the desired gene from the
 20 production host genome and replace it by the marker gene. The marker gene may be *amd S* from *Aspergillus nidulans*, *hph* from *E.coli*, or any other dominant or auxotrophic selection marker known in the literature. The vector may be a cloning vector from the pUC series or any other generally available cloning vector. The deletion cassette (comprising the DNA
 25 sequence encoding the chosen marker gene and flanking regions) is removable from the vector genome by restriction enzymes.

The present invention was exemplified by inactivating one or more DNA sequences encoding hydrophobins in fungal host strains. The inactivation of already one of the DNA sequences encoding hydrophobins significantly affected foam formation.

30 In particular, the present invention was exemplified by the deletion of *hfb1* and/or *hfb2* genes from the *Trichoderma reesei* genome. The cloning and isolation of these genes and purification of the corresponding proteins HFBI and HFBII has been described in the PhD

Thesis of Nakari-Setälä (Nakari-Setälä, T., VTT Publications 254, Espoo 1995) (Nakari-Setälä et al. 1996; Nakari-Setälä et al. 1997) In the Thesis HFBI and HFBII of *T. reesei* were speculated to play a role in attachment to substrates or to contribute to spore hydrophobicity, but the biological roles of these proteins was largely unknown before this invention. Hence
 5 their significant role in foam formation during fermentation as shown in the present invention was a surprising feature. In the present invention it was shown that the deletion of already one of the genes encoding hydrofobins in the genome of *Trichoderma reesei* resulted in remarkable decrease of foam formation during fermentation of a modified *Trichoderma* host.

10 In order to delete *hfb1* and *hfb2* genes from *Trichoderma reesei* genome, vectors comprising a deletion of these genes were constructed. In the vector *hfb1* gene was replaced by *amdS* gene and in the vector *hfb2* gene was replaced by *hph* gene of *E. coli* coding for hygromycin B. The cloning vector was one of the vectors of the pUC series, pUC 18.

15 Different *T. reesei* strains (QM 9414 and Rut-C-30) were transformed with the deletion vectors by transformation methods known in the art as described in Example 2 and the removal of the *hfb* genes in the transformants obtained was confirmed. The transformants having deletion of *hfb1*, *hfb2*, or both were cultivated in shake flasks on different growth media with glucose, sorbitol and lactose as carbon sources. The results showed that the
 20 deletion of *hfb1*, *hfb2* or both genes had no negative effect on the production of enzymes by *T. reesei*. In some cases the enzyme production was even better than with the parental *T. reesei* strain.

As a result of the *hfb1* gene deletion, the cell walls, the $\Delta hfb1$ hyphae grown on glucose in
 25 shake flasks cultivations look thinner than the control hyphae, and the strain also formed large pellets during cultivation. The growth of the transformant was somewhat impaired in the middle of the cultivation but the deletion strain reached the host strain at later stages of the cultivation. Deletion of the *hfb2* gene did not have effect on the morphology of the transformant when grown on lactose in shaken liquid cultures and no difference in growth
 30 was detected inbetween the transformant and the host strain.

T. reesei Rut C30 strain modified to have deletion of *hfb2* in its genome was also grown on lactose and cellulose in a fermentor. The consumption of the antifoam agent, pH, growth and

protein and enzyme production was monitored. The results clearly showed that deletion of a single hydrophobin gene significantly decreased the foam formation. Especially in the cultivation of *T. reesei* Rut-C30 on cellulose, in which the cultivation medium contains insoluble cellulose polymers, large amount of rather stable foam was formed and therefore antifoam consumption was high. The cultivation of the transformant on lactose did not utilise any antifoam agent and on cellulose the amount of antifoam needed during the cultivation was only 12% of that of the control cultivation. The decreased consumption of antifoam has several positive effects. The major advantage is a much easier downstream processing.

- 10 The deletion of the *hfb2* gene had no significant effect on growth of the transformant strain in fermentor in the tested conditions. Nor had the deletion of the *hfb2* gene any clear negative effect on extracellular protein and enzyme production by the transformant.

The production of a protein product was here exemplified by producing fusion molecules comprising a hydrophobin-like protein. A production host strain was modified not to produce proteins or polypeptides associated with foam formation and the same host was transformed to produce a fusion molecule comprising an amphipathic protein and a molecule of interest for further purification in aqueous two-phase system (ATPS).

- 20 The following examples are for illustration of the present invention and should not be construed as limiting the present invention in any manner.

EXAMPLES

25 Example 1

Construction of vectors for deletion of *hfb1* and/or *hfb2* genes from the *Trichoderma reesei* genome

- 30 For deletion of *hfb1* (SEQ ID 1, Figure 1) gene from *T. reesei* genome, a plasmid was constructed in which the *hfb1* coding region was replaced by the *amdS* gene of *Aspergillus nidulans* coding for acetamidase. Plasmid pEA10 (Nakari-Setälä et al. Eur. J. Biochem. (1996) 235:248-255) carrying ca. 5.8 kb *Sall* fragment containing the *hfb1* coding and

flanking regions was digested with *MunI* and blunted with T4 DNA polymerase. The subsequent vector fragment missing the ca. 950 bp *MunI* fragment containing the coding and some flanking regions for *hfb1* was purified from agarose gel and ligated to a 3.2 kb *amdS* fragment released from p3SR2 (Hynes et al. Mol. Cell Biol. (1983) 3:1430-1439; Tilburn et al. Gene (1983) 26:205-221) with *SphI* and *XbaI* and blunted with T4 DNA polymerase. The resulting plasmid is pTNS24 (Figure 3). It carries a deletion cassette containing the *amdS* gene with ca. 2.7 kb and 2 kb of the *hfb1* 5' and 3' non-coding regions, respectively. The deletion cassette may be released from the vector with *Sall*.

10 For removal of the *hfb2* (SEQ ID 2, Figure 2) gene from *T. reesei* genome, a plasmid was constructed in which the *hfb2* coding region was replaced by the *hph* gene of *E. coli* coding for hygromycin B phosphotransferase. A 1.2 kb 5' flanking region of the *hfb2* gene was released from the plasmid pTNS8 (Nakari-Setälä et al. Eur. J. Biochem. (1997) 248:415-423) with *HindIII* and *BglI*, blunted with T4 DNA polymerase and purified from agarose gel. The purified fragment was ligated to pARO21 (Penttilä et al. Patent appl. FI 990667) cut with *XhoI* and blunted with T4 DNA polymerase resulting in pTNS26. pARO21 is essentially the same as pRLMex30 (Mach et al. Curr. Genet. (1994) 6:567-570) and carries the *E. coli hph* gene operably linked to 730 bp of *pkil* promoter and 1 kb of *cbh2* terminator sequences of *T. reesei*. To introduce the 3' flanking region of *hfb2* into the deletion cassette, pTNS26 was cut with *EcoRV*, treated with SAP (shrimp alkaline phosphatase, Boehringer-Mannheim) and ligated to a 1.7 kb *EcoRV hfb2* 3' flanking region fragment released from pTNS8. In the resulting plasmid pTNS27 (Figure 4) both the 5' and 3' non-coding regions of the *hfb2* gene flank the *hph* expression cassette in the same orientation. The deletion cassette may be released from the vector with *SphI* and *SpeI*.

Example 2

Transformation of *Trichoderma* and purification of Δhfb clones

Trichoderma reesei strains QM9414 (VTT-D-74075), Rut-C30 (VTT-D-86271) and QM9414 $\Delta hfb1$ (VTT-D-99724) were transformed essentially as described (Penttilä et al., Gene (1987) 61:155-164) using 3-13 μ g of the plasmids pTNS24 and pTNS27 or the deletion cassettes released from them with the proper restriction enzymes.

The Amd⁺ and Hyg⁺ transformants obtained were streaked three times onto plates containing acetamide and hygromycin, respectively (Penttilä et al. (1987) Gene 61:155-164). Thereafter spore suspensions were made from transformants grown on Potato Dextrose agar (Difco).

- 5 To confirm the removal of the *hfb* genes from the genomes, Southern analyses were carried out. Mixed Amd⁺ and Hyg⁺ transformants were cultivated on minimal medium (Penttilä et al. (1987) Gene 61:155-164) containing 3% glucose and 0.2% peptone for isolation of genomic DNA using Easy-DNA kit (Invitrogen). Approximately 2 µg of DNA were cleaved with PvuI in the case of $\Delta hfb1$ transformants and with NcoI in the case of $\Delta hfb2$ transformants. Southern
- 10 hybridizations with ca. 5.8 kb and 3.8 kb genomic fragments containing *hfb1* and *hfb2* genes and flanking regions were carried out. In the host strain, the hybridization with the probes results in both cases in one large signal whereas in transformants two smaller signals are obtained if the *hfb* gene has been correctly deleted.
- 15 The spore suspensions of the clones from which the *hfb* genes had been deleted on the basis of Southern analysis were purified to single spore cultures on selection plates (containing either acetamide or hygromycin). Southern analysis was repeated similarly as above to select pure Δhfb clones.
- 20 *T. reesei* strains used for further studies are VTT-D-99724 and VTT-D-99723 (QM9414 $\Delta hfb1$), VTT-D-99726 (QM9414 $\Delta hfb2$), VTT-D-99725 (QM9414 $\Delta hfb1\Delta hfb2$) and VTT-D-99676 (Rut-C30 $\Delta hfb2$).

Example 3

25

Enzyme production of *Trichoderma* QM9414 of $\Delta hfb1$, $\Delta hfb2$ and $\Delta hfb1\Delta hfb2$ strains on glucose, sorbitol and lactose

- 30 Strains VTT-D-99724 ($\Delta hfb1$), VTT-D-99726 ($\Delta hfb2$), VTT-D-99725 ($\Delta hfb1\Delta hfb2$) and their host strain VTT-D-74075 (QM9414) were cultivated in shake flasks at 28°C for three and seven days in 50 ml of *Trichoderma* minimal medium (Penttilä et al. 1987) supplemented with 0.2% peptone and as a 2% carbon source either i) glucose, ii) sorbitol or iii) lactose. Starting pH was pH 4.8. Culture medium samples were taken after 3 of cultivation. Secreted

total proteins were analysed by the method of Lowry et al. 1951. Endoglucanase and endoxylanase activities were measured according to IUPAC standard method and Bailey et al. (1992) using hydroxyethyl cellulose (HEC, Fluka 54290) and birch xylan (XYL, Roth 7500) as substrates, respectively. Protease activity was determined semi-quantitatively by dotting 5 μ l of culture filtrates on 1.5% agar plates (pH 5) containing 1% skim milk and estimating the size of the halos due to protease activity in the samples.

The values obtained from activity measurements and calculated as enzyme activity per total secreted protein are presented in the table below. These figures indicate that deletion of *hfb* genes has no negative, but in some cases even positive, effect on production of both glycanases and proteanases, two groups of enzymes whose expression is under different regulatory mechanisms. Culture broth pH values at the end of the cultivation indicate that on the same culture medium all strains are approximately in the same growth stage. No clear differences were seen visually in morphology or amount of cell mass in between the different strains on same medium.

Strain	Carbon source	HEC nkat/mg total protein	XYL nkat/mg total protein	Protease
Host QM9414	Glucose	nd	nd	+
$\Delta hfb1$		nd	nd	+++
$\Delta hfb2$		nd	nd	+
$\Delta hfb1\Delta hfb2$		nd	nd	++
Host QM9414	Sorbitol	-	30	-
$\Delta hfb1$		-	50	-
$\Delta hfb2$		-	60	-
$\Delta hfb1\Delta hfb2$		-	63	-
Host QM9414	Lactose	78	253	-
$\Delta hfb1$		98	202	-
$\Delta hfb2$		89	363	-
$\Delta hfb1\Delta hfb2$		92	190	-

nd, not determined

-, not detected

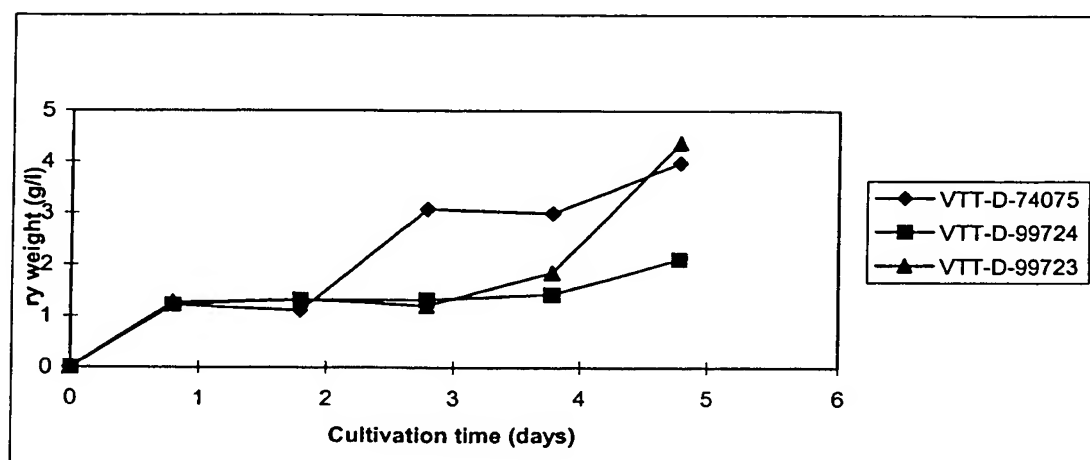
Example 4

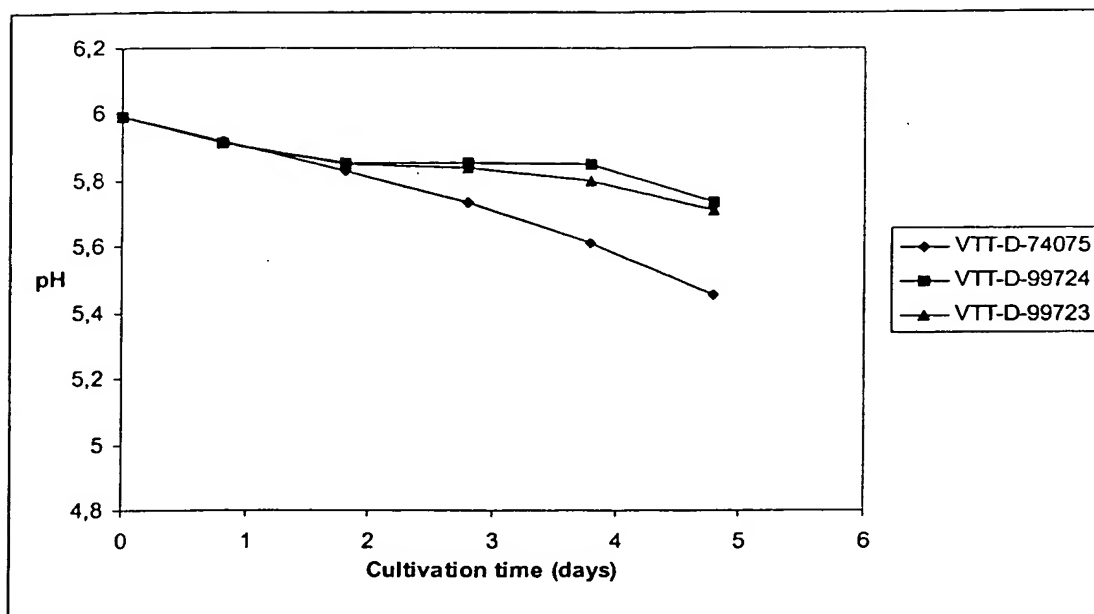
Growth and morphology of *T. reesei* QM9414 $\Delta hfb1$ strain on glucose

5 The strains VTT-D-99724 ($\Delta hfb1$) and VTT-D-99723 ($\Delta hfb1$) originating from two independent transformants and their host strain VTT-D-74075 (QM9414) were also cultivated in shake flasks at 28°C for five days in 250 ml of *Trichoderma* minimal medium (Penttilä et al. 1987) buffered to pH 6 and supplemented with 3% glucose. The growth of the strains was followed by measuring the mycelial dry weights and qualitatively from the pH values of
10 culture media at different time points.

As a result of the *hfb1* gene deletion, the ability of *T. reesei* to grow on glucose was impaired in shaken liquid cultures as can be seen from the mycelial dry weight and pH figures presented below. The appearance of the hyphae in the glucose cultivation was monitored with
15 a light microscope to study the morphology of the deletion strains. Probably due to the lack of HFBI protein in the cell walls, the $\Delta hfb1$ hyphae looked thinner than the control hyphae, and the strain also formed large pellets during cultivation. However, it should be noted that during the time course of the cultivation the deletion strain VTT-D-99723 reaches the host strain in terms of biomass production.

20





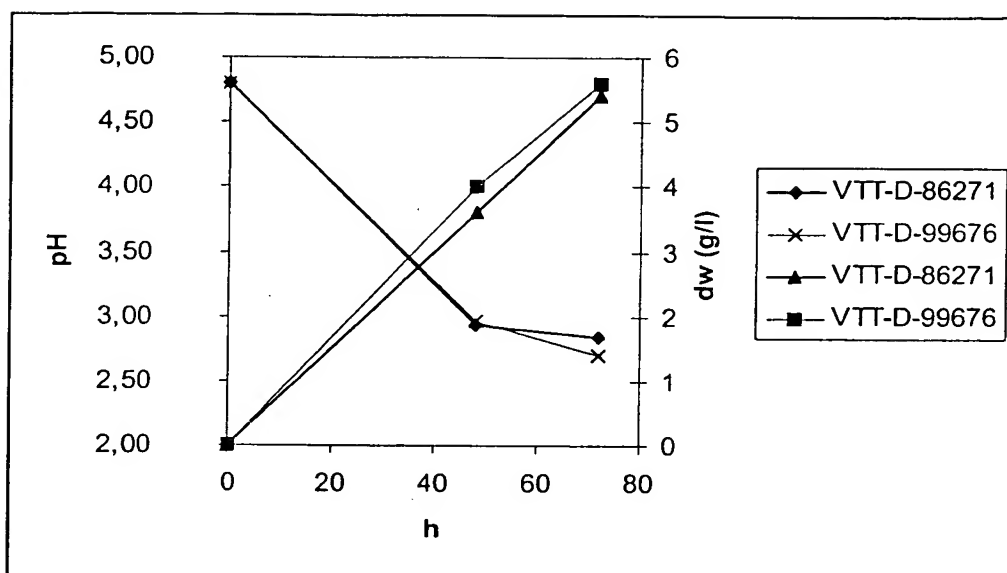
Example 5

Cultivation of *Trichoderma* Rut C30 $\Delta hfb2$ strain on lactose in shake flasks

5

Strain VTT-D-99676 ($\Delta hfb2$) and its host strain VTT-D-86271 (Rut-C30) were cultivated at 28°C in shake flasks for three days in 250 ml of *Trichoderma* minimal medium (Penttilä et al. 1987) buffered to pH 6 and supplemented with 0.2% peptone and 2% lactose with lactose feeding. Samples were taken from the flasks on days 2 and 3 for analysis of growth and protein production. Growth was analysed quantitatively by measuring the mycelial dry weight and qualitatively from pH values of culture medium. No differences in growth were detected inbetween the transformant and the host strain on the basis of these measurements as seen from the figure below. Also, the morphology of the transformant strain was similar to that of the host strain.

15



Production levels of secreted endoglucanases (HEC) and cellobiohydrolases (MUL) measured according to IUPAC standard method and Tilbeurgh et al. (1988), respectively, are presented in the table below. These results clearly show that the deletion of the *hfb2* gene from the control strain had no negative effect on enzyme production.

Strain	h	dry weight g/l	HEC nkat/ml	MUL nkat/ml
VTT-D-86271	0	0	0	0
	48	3.6	230	3.2
	72	5.4	247	9
VTT-D-99676	0	0	0	0
	48	4	236	2.7
	72	5.6	214	7

10

Example 6

Cultivation of *Trichoderma* Rut C30 $\Delta hfb2$ strain on lactose in fermenter

Modern cellulose production media may for some, but not all strains be based on lactose as the carbon source and enzyme inducer. The control strain Rut-C30 produces cellulases efficiently on lactose, whereas e.g. the strain QM9414 does not.

Trichoderma reesei Rut-C30 and its $\Delta hfb2$ transformant VTT D-99676 (D-676) were cultivated on lactose-based medium in a 15 litre laboratory fermenter. The medium contained (in g l⁻¹) lactose (Riedel-de Haën, Germany, product 33411) 40, peptone (Difco, USA, 0118-17) 4.0, yeast extract (Difco, 0127-17), 1.0, KH₂PO₄ 4.0, (NH₄)₂SO₄ 2.8, MgSO₄·7H₂O 0.6, CaCl₂·2H₂O 0.8 (sterilised separately) and 2.0 ml l⁻¹ of a trace solution (Mandels and Weber 1969). No antifoam agent was added to the medium before the in-situ sterilisation (123°C/20 min). Cultivation conditions were: temperature 29°C, agitation 600 rpm, aeration 10 l min⁻¹, pH 4.0...5.0.

10

In these cultivations on the completely soluble medium, it was possible to monitor growth by analysis of biomass dry weight and by consumption of lactose. A comparison of the growth parameters in the two cultivations is presented in Figures [8] A and B. The results clearly showed that deletion of the *hfb2* gene from the control strain had no significant effect on growth. Foaming was not a major problem on this medium with either strain. Consumption of antifoam agent was 10 ml/15 litres with strain Rut-C30, whereas no antifoam agent at all was consumed in the cultivation of strain D-676.

In this pair of cultivations, cellulase production by the $\Delta hfb2$ strain was somewhat lower than by the control strain Rut-C30 (Table below). However, experience with cellulase production on lactose-based media has shown that optimisation of process conditions must be performed for each producing strain separately. The effects of incipient catabolite repression due to cleavage of the lactose molecule to glucose and galactose must be avoided by suitable, strain-specific adjustment of pH and/or temperature.

25

Table. Production of soluble protein, cellulases (HEC, FPU, IUPAC standard method) and overall consumption of antifoam agent (Struktol J633) in cultivations of *T. reesei* Rut C30 and its $\Delta hfb2$ transformant VTT D-99676 on lactose medium in a 15 litre laboratory fermenter.

30

Strain	Dry weight g l ⁻¹	Protein g l ⁻¹	HEC nkat ml	FPU u ml ⁻¹	Antifoam consumption ml l ⁻¹
Rut-C30	15.8	5.9	780	3.5	0.7
VTT D-99676	14.6	5.0	610	2.1	0.0

Example 7

Cultivation of *Trichoderma* Rut C30 $\Delta hfb2$ strain on cellulose in fermenter

5

Trichoderma reesei Rut C30 and its $\Delta hfb2$ transformant VTT D-99676 (D-676) were cultivated on cellulose-spent grain medium in a 15 litre laboratory fermenter. The medium contained (in g l⁻¹) Solka floc cellulose 40, distiller's spent grain 20, KH₂PO₄ 5 and (NH₄)₂SO₄ 5. A standard dose of 5 ml antifoam agent (Struktol J633, Schill&Seilacher, Hamburg, Germany) was added to the medium to prevent foaming during the in-situ sterilisation (123°C/20 min). Cultivation conditions were: temperature 29°C, agitation 600 rpm, aeration 10 l min⁻¹, pH 4.0...5.0.

Growth in terms of biomass production or substrate consumption could not be measured on the solids-based medium, but production curves of cellulase (HEC; FPU) and soluble protein (Lowry et al. 1951) indicated rather similar rates and levels of production by the control and transformant strains (Figures [9] A and B). The curves of broth pH are also presented in the figure as an indication of the sequence of growth phases: first indeterminate or increasing trend during the lag phase and early growth, decreasing pH during the main growth phase and finally an increasing trend during secondary metabolism/starvation. Production levels of the cellulase and xylanase activities measured in the cultivations are presented in Table below. These results clearly show that deletion of the *hfb2* gene from the control strain had no negative effect on enzyme production. Due to the unhomogenous nature of all the enzyme substrates used in this comparison, typical variation in analysis results is in the region of $\pm 10\%$ for HEC and XYL and at least 15-20% for FPU.

Table. Production of soluble protein, cellulases (HEC, FPU, IUPAC standard method) and xylanase (XYL, Bailey et al. 1992) and overall consumption of antifoam agent (Struktol J633) in cultivations of *T. reesei* Rut C30 and its $\Delta hfb2$ transformant VTT D-99676 on cellulose-spent grain medium in a 15 litre laboratory fermenter.

5

Strain	Protein g l ⁻¹	HEC nkat ml	FPU u ml ⁻¹	XYL nkat ml ⁻¹	Antifoam consumption ml l ⁻¹
Rut C30	13.9	1600	10.9	1870	3.3
VTT D-99676	16.4	1715	8.9	2240	0.4

The major, striking difference between the two cultivations was in the consumption of antifoam agent: in the control cultivation with strain Rut C30 consumption of Struktol was 50
10 ml/15 litres, whereas in the case of strain D-676 only 6.0 ml was consumed. Apparently the latter cultivation foamed only on one occasion, towards the very end of the run. This foaming was presumably the result of the secretion of enzyme proteins into the medium, which was obviously independent of the presence or absence HFBII. The almost tenfold difference in the requirement for antifoam agent would certainly have a significant effect on industrial scale
15 downstream processing (enzyme concentration by membrane filtration and possible chromatographic purification).

Example 8

Cultivation of $\Delta hfb1\Delta hfb2$ *Trichoderma* Rut-C30 strain on glucose in fermenter

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Trichoderma reesei strain QM9414 (VTT-D-74075) and its $\Delta hfb1\Delta hfb2$ transformant VTT D-99725 is cultivated on glucose-based medium in a 15 litre laboratory fermenter. The medium contains (in g l⁻¹) glucose, 40, peptone (Difco, USA, 0118-17) 4.0, yeast extract (Difco, 0127-17), 1.0, KH₂PO₄ 4.0, (NH₄)₂SO₄ 2.8, MgSO₄·7H₂O 0.6, CaCl₂·2H₂O 0.8 (sterilised separately) and 2.0 ml l⁻¹ of a trace solution (Mandels and Weber 1969). No antifoam agent is
25 added to the medium before the in-situ sterilisation (123°C/20 min). Cultivation conditions are: temperature 29°C, agitation 600 rpm, aeration 10 l min⁻¹, pH 4.0...5.0.

Morphology, biomass dry weight, pH, oxygen and antifoam agent consumption and production of extracellular protein (Lowry et al. 1951) and protease activity is analysed during the cultivations.

5

Example 9

Production of EGIcore-HFBI fusion proteins in *T. reesei* $\Delta hfb2$ strain for improved partitioning of the fusion protein in ATPS

10

For construction of an EGIcore-HFBI fusion protein, the *hfb1* coding region from Ser-23 to the STOP codon was amplified with PCR with the following primers, as a 5' primer **ACT ACA CGG AG G AGC TC G ACG ACT TCG AGC AGC CCG AGC TGC ACG CAG** AGC AAC GGC AAC GGC (SEQ ID No 3) and as a 3' primer TCG TAC GGA TCC TCA AGC ACC GAC GGC GGT. (SEQ ID No 4) The sequence in bold in the 5' primer encodes amino acids 410-425 in EGI and the underlined GAGCTC is a SacI site. The 260 bp PCR fragment was purified from agarose gel and ligated to pPCRII T/A vector (Invitrogen) resulting in pMQ111.

20 In the next step *Trichoderma* expression vectors for production of EGIcore-HFBI fusion protein under the control of *cbh1* promoter and terminator sequences was constructed. The expression vector used as a backbone in the constructs is pPLE3 (Nakari et al. (1994) WO 94/04673) which contains a pUC18 backbone, and carries the *cbh1* promoter inserted at the EcoRI site. The *cbh1* promoter is operably linked to the full length *egl* cDNA coding sequence and to the *cbh1* transcriptional terminator. The plasmid pMQ111 was digested with 25 SacI and BamHI and the 260 bp fragment containing the *hfb1* sequence was ligated to pPLE3 digested with SacI and BamHI. The resulting plasmid pMQ113 (Figure 5) carry the coding sequences for EGIcore linked to HFBI via its own linker region under the control of *cbh1* promoter and terminator sequences.

30

Trichoderma reesei strain QM9414 $\Delta hfb2$ (VTT-D-99726) was transformed essentially as described (Penttilä et al., Gene (1987) 61:155-164) using 10 μ g of the plasmid pMQ113 together with 3 μ g of the selection plasmid pTOC202 containing the *amdS* gene (Hynes et al.

Mol. Cell Biol. (1983) 3:1430-1439; Tilburn et al. Gene (1983) 26:205-221) of *Aspergillus nidulans* encoding for acetamidase.

The Amd+ transformants obtained were streaked two times onto plates containing acetamide
5 (Penttilä et al. (1987) Gene 61:155-164). Thereafter spore suspensions are made from transformants grown on Potato Dextrose agar (Difco). The production of the EGIcore-HFBI fusion protein is tested by slot blotting or Western analysis with EGI and HFBI specific antibodies from shake flask or microtiter plate cultivations carried out in minimal medium supplemented with a mixture of Solka floc cellulose and/or spent grain and/or whey. The
10 spore suspensions of the clones producing fusion protein are purified to single spore cultures on selection plates (containing acetamide). To determine the best producers, production of the fusion protein is analyzed again from these purified clones as described above.

For partitioning experiments of the EGIcore-HFBI fusion protein in ATPS using the
15 polyoxyethylene detergent C₁₂₋₁₈EO₅ (Agrimul NRE 1205, Henkel) the best production strain obtained in this study and as control strains VTT-D-98691 (QM9414 strain producing EGIcore-HFBI), VTT-D-74075 (QM9414) and VTT-D-99726 (QM9414 $\Delta hfb2$) are cultivated at 28°C in shake flasks for 5 to 6 days in 50 to 250 ml volume of *Trichoderma* minimal medium (Penttilä et al. 1987) supplemented with 3% Solka floc cellulose and 1%
20 spent grain.

Partitioning experiments are carried out with supernatant (biomass separated by centrifugation or filtration) in 10 ml graduated tubes. First detergent is added into the tubes and the tubes are then filled to 10 ml with culture supernatant. The amount of detergent in the tube is calculated
25 in weight percent of detergents. After thorough mixing in an overhead shaker the separation takes place by either gravity settling in a water bath at constant temperature or by centrifugation at constant temperature. The separation is usually performed at 30°C, the standard amount of detergent used is 2-5% (w/v). After separation the volume ratio of the lighter and heavier phase is noted and the concentration factor for the fusion protein is
30 calculated from it. Samples are also taken from the lighter and heavier phase for analysis.

Two-phase separations are analysed qualitatively by using SDS-PAGE gels followed by visualization of the fusion proteins with Coomassie brilliant blue R-250 (Sigma) or Western

blotting. Polyclonal anti-HFBI antibody are used in Western analysis for detection of EGIcore-HFBI protein together with alkaline phosphatase conjugated anti-rabbit IgG (Bio-Rad). Alkaline phosphatase activity is detected colorimetrically with BCIP (5-bromo-4-chloro-3-indolyl-phosphate) used in conjunction with NBT (nitro blue tetrazolium) (Promega).

EGI activity is detected using 4-methylumbelliferyl- β -D-cellobioside (MUC) (Sigma M 6018) as substrate (Van Tilbeurgh H. & Caeyssens M., 1985; Van Tilbeurgh *et.al.*, 1982). EGI hydrolyses the β -glycosidic bond and fluorogenic 4-methylumbelliferone is released, which can be measured using a fluorometer equipped with a 360 excitation filter and a 455 nm emission filter. CBHI also hydrolyses the substrate and it is inhibited by addition of cellobiose (C-7252, Sigma). EGI containing liquid is added in an appropriate dilution to a buffer containing 50 mM sodium acetate buffer (pH 5), 0.6 mM MUC and 4.6 mM cellobiose. The mixture is heated to 50°C. The reaction is stopped after ten minutes using 2% Na₂CO₃, pH 10. Purified CBHI is detected using the same assay as for EGI without the addition of the inhibitor cellobiose.

The partition coefficient K is defined as the ratio of the measured concentrations or activities in the top and bottom phase respectively.

The Yield Y is defined as follows:

$$Y_T = \frac{1}{1 + \left[\frac{V_B}{V_T} \cdot \frac{1}{K} \right]}$$

where Y_T is the Yield of the top phase, V_B and V_T are the volumes of top and bottom phase respectively. The Yield of the bottom phase can be described accordingly.

The mass balances, e.g. recovery of all added protein, are always checked for completeness to ensure no artificially high Yield (e.g. due to possible inactivation of the protein in the bottom phase). The values are usually calculated based on total enzyme activity (EGI wt plus the EGI-fusion) and thus the values are underestimated for the separation of the fusion.

Example 10

Production of HFBI-single chain antibody fusion protein in *T. reesei* $\Delta hfb2$ strain for purification in ATPS

5

A *T. reesei* strain is constructed which produces a fusion protein consisting of *T. reesei* HFBI protein in the N-terminus and in the C-terminus a single chain antibody (ENA5SCFV) recognizing a small molecular weight derivative of diarylalkyltriazole. The fusion is to be subjected for purification using aqueous two-phase system.

10

For cloning of the enantiospecific Fab-fragments against the diarylalkyltriazole antigen, total RNA was isolated using the extraction protocol of Promega from the hybridoma cell line obtained from the spleen cells of mice immunized with antigen. The mRNA fraction was purified using the Oligotex-dT polyA⁺ purification kit of

15

Qiagen and the cDNA synthesis was performed with AMV reverse transcriptase - system of Promega. The cDNA was then subjected to PCR amplification using primer mixtures specific for each antibody group, 9 for heavy chains and 4 for light chains (according to Kabat *et al.*, 1991, Sequences of proteins of immunological interest, NIH publication No. 91-3242). The polymerization was carried out using the

20

Dynazyme polymerase (Finnzymes) and standard conditions according to the manufacturer's instructions. After the amplification, both the heavy and the light chains were cloned as a dicistronic operon under the tac promoter controlled by the *lacI*^q repressor present in the expression vector pTI8 (Takkinen *et al.*, 1991). For secretion, the PelB signal sequence of pectate lyase of *Erwinia carotovora* (Takkinen

25

et al., 1991) was linked for both the heavy and the light chains. A six histidine tag was added to the C-termini of the light chains. The resulting plasmid is pENA5-His.

30

For construction of a ENA5SCFV single chain antibody, the variable domains of the heavy and light chains were amplified with PCR using pENA5-His as template. The amplified fragment was cloned into pKKtac vector (Takkinen *et al.* 1991) resulting in pENA5SCFV. pENA5SCFV vector carries the coding region for ENA5SCFV single chain antibody consisting of the variable domains of the heavy and light chains connected via a glycine serine linker (Huston *et al.* 1988 and 1991) and a 6 x histidine

tag at the C-terminal end. Transcription and secretion of the single chain antibody are under control of the *tac* promoter and *pelB* signal sequence, respectively (Takkinen et al. 1991).

- 5 For construction of HFBI-ENA5SCFV fusion protein, pENA5SCFV was digested with NcoI and XbaI. The fragment containing the *ena5scfv* gene and the histidine tail (6 x His) was blunt-end cloned to pTNS29 resulting in pTH1 (Figure 6). pTNS29 vector carries the *hfb1* coding region of *T. reesei* followed by a linker sequence (ProGlyAlaSerThr SerThrGlyMetGlyProGlyGly) (SEQ ID No 5) under the control of
10 *cbh1* promoter and terminator sequences.

For construction of HFBI-ENA5SCFV fusion protein with a thrombin cleavage site in the linker peptide, *ena5scfv* coding region (from Ala-23 to the STOP codon) and a peptide linker containing the thrombin cleavage site (Gly Thr Leu Val Pro Arg Gly
15 Pro Ala Glu Val Asn Leu Val) (SEQ ID No 6) preceding it was amplified with PCR using pENA5SCFV as a template and as a 5' primer GAA TTC GGT ACC CTC GTC CCT CGC GGT CCC GCC GAA GTG AAC CTG GTG (SEQ ID No 7) and as a 3' primer TGA ATT CCA TAT GCT AAC CCC GTT TCA TCT CCA G (SEQ ID No 8). The sequence in bold in the 5' primer encodes the first 6 N-terminal residues of
20 ENA5SCFV. The sequence in italics is a thrombin cleavage site and underlined GGT ACC is Asp718 site. The sequence in bold in the 3' primer encodes the 6 C-terminal residues of ENA5SCFV and the underlined CA TATG is a NdeI site. The 790 bp PCR fragment was purified from agarose gel and ligated to pTNS29 resulting in pTH2 (Figure 7).

25 *Trichoderma reesei* strain VTT-D-99726 (QM9414 $\Delta hfb2$) is co-transformed essentially as described (Penttilä et al., Gene (1987) 61:155-164) using 10 μ g of the plasmids pTH1 and PTH2 and as selection plasmid 2 μ g pTOC202. Amd+ transformants obtained are streaked two times onto plates containing acetamide.
30 Thereafter spore suspensions are made from transformants grown on Potato Dextrose agar (Difco).

The production of the two HFBI-ENA5SCFV fusion proteins is tested by Western analysis with HFBI specific antibody and with the antibody against the his-tail from shake flask cultivations carried out in minimal medium supplemented with 3 % lactose or Solka flock cellulose and spent grain.

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Partitioning experiments of the HFBI-ENA5 fusion proteins in ATPS using the polyoxyethylene detergent C₁₂₋₁₈EO₅ (Agrimul NRE 1205, Henkel) with the supernatants of the best production strains obtained in this study and the control strain VTT-D-99726 (QM9414 $\Delta hfb2$) are carried out and analysed as described in Example 9.

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References

- Arntz, C. and Tudzynski, P. (1997) Identification of genes induced in alkaloid-producing cultures of *Claviceps* sp.. *Curr. Genet.* 31: 357-360.
- Bailey, M.J., Biely, P. and Poutanen, K. (1992) Interlaboratory testing of methods for assays
5 of xylanase activity. 23:257-270.
- Cameotra, S.S. and Makkar, R.S. (1998) Synthesis of biosurfactants in extreme conditions. *Appl. Microbiol. Biotechnol.* 50:520-529.
- Carpenter, C.E., Mueller, R.J., Kazmierczak, P., Zhang, L., Villalon, D.K. and VanAlften, N.K. (1992) Effect of a virus on accumulation of a tissue-specific cell surface protein of the
10 fungus *Cryphonectria (Endothia) parasitica*. *Mol. Plant-Microbe Interact.* 4:55-61.
- de Vries, O.M.H., Fekkes, M.P., Wösten, H.A.B. and Wessels, J.G.H. (1993) Insoluble hydrophobin complexes in the walls of *Schizophyllum commune* and other filamentous fungi, *Arch Microbiol.* 159:330-335.
- de Vries, O.M.H., Moore, S., Arntz, C., Wessels, J.G.H. and Tudzynski, P. (1999)
15 Identification and characterization of a tri-partite hydrophobin from *Claviceps fusiformis* Eur. J. Biochem. 262:377-385.
- Huston, J.S. et al. (1988) *Proc. Natl. Acad. Sci. USA.* 85:5879-5883.
- Huston, J.S., Mudgett-Hunter, M., Tai, M.-S., McCartney, J., Warren, F., Haber, E. and Oppermann, H. (1991) *Methods Enzymol.* 203: 46-88.
- 20 Hyytiä, T., Selber, K., Qiao, M., Fagerström, R., Kula, M.-R., Nakari-Setälä, T. and Penttilä, M., (1999). A novel strategy for protein production and purification in *Trichoderma reesei*. 6th International *Trichoderma-Gliogladium* Workshop. June 11-13, 1999. Hanasaari, Finland.
- Kaartinen, M., Knowles, J.K.C. and Teeri, T.T. (1991) An active single-chain antibody containing a cellulase linker domain is secreted by *Escherichia coli*. *Protein Eng.* 4:837-841
- 25 Kershaw, M.J. and Talbot, N.J. (1998) Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis. *Fungal Gen. Biol.* 23:18-33.
- Lang, S. and Wullbrandt, D. (1999) Rhamnose lipids – biosynthesis, microbial production and application potential. *Appl. Microbiol. Biotechnol.* 51:22-32.
- Lora, J.M., de la Cruz, J., Betitez, T., Liobell, A. and Pintor-Toro, J.A. (1994) A putative
30 catabolite-repressed cell wall protein from the mycoparasitic fungus *Trichoderma harzianum*. *Mol. Gen. Genet.* 242:461-466.
- Lora, J.M., Pintor-Toro, J.A., Benitez, T. and Romero, L.C.(1995) *Mol. Microbiol.* 18:377-382.

Lowry, O.H., Rosebrough, N.H., Parr, A.L. and Randell, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275

IUPAC (1997) Measurement of cellulase activities. *Pure Appl. Chem.* 59:257-268

Muñoz, G., Nakari-Setälä, T., Agosin, E. and Penttilä, M. (1997). Hydrophobin gene *srh1*,

5 expressed during sporulation of the biocontrol agent *Trichoderma harzianum*. *Curr. Genet.* 32:225-230.

Nakari-Setälä, T., Aro, N., Kalkkinen, N., Alatalo, E. and Penttilä, M. (1996). Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFBI. *Eur. J. Biochem.* 235:248-255.

10 Nakari-Setälä, T., Aro, N., Ilmén, M., Muñoz, G., Kalkkinen, N. and Penttilä, M. (1997). Differential expression of the vegetative and spore-bound hydrophobins of *Trichoderma reesei*: cloning and characterization of the *hfb2* gene. *Eur. J. Biochem.* 248:415-423.

Richter, M., Willey, J.M., Süssmuth, R., Jung, G. and Fiedler, H.-P. (1998) Streptofactin, a novel biosurfactant with aerial mycelium inducing activity from *Streptomyces tendae*. *FEMS*

15 *Microbiol Lett* 163:165-171.

Schuren, F.H.J. and Wessels, J.G.H. (1990) Two genes specifically expressed in fruiting dikaryons of *Schizophyllum commune*: homologues with gene not regulated by mating-type genes. *Gene* 90:199-205.

Russo, P.S., Blum, F.D., Ipsen, J.D., Miller, W.G. and Abul-Haki, Y.J. (1992) The surface activity of the phytotoxin cerato-ulmin. *Can. J. Bot.* 60:1414-1422.

20 Takkinen, K., Laukkanen, M.-L., Sizmann, D., Alfthan, K., Immonen, T., Vanne, L., Van der Vegt, W., van der Mei, H.C., Wösten, H.A.B., Wessels, J.G.H. and Busscher, H.J. (1996) A comparison of the surface activity of the fungal hydrophobin SC3p with those of other proteins. *Biophys Chem.* 51:253-260.

25 Van Tilbeurgh, H., Loontjens, F.G., De Bruyne, C.K. and Clayessens, M. (1988). *Methods Enzymol.* 160:45-59.

Wessels, J.G.H. (1994) Developmental regulation of fungal cell wall formation. *Ann. Rev. Phytopathol.* 32:413-437.

Wessels, J.G.H. (1997). Hydrophobins: proteins that change the nature of the fungal surface. 30 *Adv. Microbial Physiol.* 38:1-45.

Wessels, J.G.H., de Vries O.M.H., Åsgeirsdóttir, S.A. and Schuren F.H.J. (1991a) Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in *Schizophyllum commune*. *Plant Cell* 3:793-799.

- Wessels, J.G.H., de Vries O.M.H., Åsgeirsdottir, S.A. and Springer, J. (1991b) The *thn* mutation of *Scizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the Sc3 hydrophobin gene. *J.Gen.Microbiol.* 137:2439-2445.
- Wiley, J.M., Santamaria, R., Guijarro, J., Geislich, M. and Losick, R. (1991) Extracellular
 5 complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicor*. *Cell* 65:641-650.
- Wösten, H.A.B., de Vries, O.M.H. and Wessels, J.G.H. (1993) Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. *Plant Cell* 5:1567-1574.
- Wösten, H.A.B., Schuren, F.H.J. and Wessels, J.G.H. (1994a) Interfacial self-assembly of a
 10 hydrophobin into an amphipathic membrane mediates fungal attachment to hydrophobic surfaces. *EMBO J* 13:5848-5854.
- Wösten, H.A.B., Asgeirsdottir, S.A., Krook, J.H., Drenth, J.H.H. and Wessels, J.G.H. (1994b) The fungal hydrophobin S'C3p self-assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobic rodlet layer. *Eur. J. Cell. Biol.* 63:122-129.
- 15 Wösten, H.A.B. and Wessels, J.G.W (1997) Hydrophobins, from molecular structure to multiple functions in fungal development. *Mucoscience* 38:363-374.
- Wösten, H.A.B., van Wetter, A.-M., Lugones, L.G., van der Mei, H.C., Busscher, H.J. and Wessels, J.G.H. (1999) How a fungus escapes the water to grow into the air. *Current Biology* 9:85-88.

CLAIMS

1. A method for decreasing the foam formation during cultivation of a microorganism, characterized in that the process comprises the steps of
 - 5 - modifying the microorganism in such a way that the microorganism does not produce an essential amount of at least one of the proteins, polypeptides or peptides associated with foam formation during cultivation; and
 - cultivating the microorganism under suitable culture conditions.
- 10 2. The method of claim 1, characterized in that the proteins, polypeptides or peptides associated with foam formation during cultivation are selected from the group comprising amphipathic or hydrophobic proteins, polypeptides or peptides or proteins, polypeptides or peptides associated or covalently linked to lipids.
- 15 3. The method of claim 1 or 2, characterized in that the proteins, polypeptides or peptides associated with foam formation are hydrophobins.
4. The method claim 3, characterized in that the hydrophobins are HFB I and/or HFBII of *Trichoderma*.
- 20 5. The method of any one of claims 1 to 4, characterized in that the modification comprises genetic modification of the microorganism.
- 25 6. The method of claim 5, characterized in that the genetic modification comprises genetic modification of a DNA sequence encoding a protein, polypeptide or peptide regulating the production of at least one of the proteins, polypeptides or peptides associated with foam formation.
- 30 7. The method of claim 5, characterized in that the genetic modification comprises genetic modification of the regulatory region of a gene encoding at least one of the proteins, polypeptides or peptides associated with foam formation

8. The method of claim 5, characterized in that the genetic modification comprises genetic modification of a DNA sequence encoding at least one of the proteins, polypeptides or peptides associated with foam formation.

5 9. The method of claim 8, characterized in that the genetic modification comprises inactivation of a DNA sequence encoding at least one of the proteins, polypeptides or peptides associated with foam formation.

10. The method of claim 9, characterized in that the genetic modification comprises
10 deletion of a DNA sequence encoding at least one of the proteins or polypeptides or peptides associated with foam formation.

11. A method for producing a product by cultivating a microorganism,
characterized in that the process comprises the steps of

- 15 - modifying the microorganism in such a way that the microorganism does not produce an essential amount of at least one of the proteins, polypeptides or peptides associated with foam formation during cultivation;
- cultivating the microorganism under suitable culture conditions; and
- recovering the product from the cultivation.

20 12. A product produced by the method of claim 11.

13. The product of claim 12, characterized in that it is a protein or a metabolite or biomass.

25 14. The product of claim 12, characterized in that it is a recombinant product.

15. A production host strain, characterized in that the host strain is genetically modified not to produce an essential amount of at least one of the proteins, polypeptides or
30 peptides associated with foam formation during cultivation of the non-modified production host strain.

16. The production host strain of claim 15, c h a r a c t e r i z e d in that the proteins or polypeptides or peptides associated with foam formation during cultivation are selected from the group comprising amphipathic or hydrophobic proteins, polypeptides or peptides or proteins, polypeptides or peptides associated or covalently linked to lipids.

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17. The production host strain of claim 16, c h a r a c t e r i z e d in that the proteins, polypeptides or peptides associated with foam formation are hydrophobins.

18. The production host strain of any one of claims 15 to 17, c h a r a c t e r i z e d in that the
10 strain is a fungal strain.

19. The production host strain of claim 18, c h a r a c t e r i z e d in that the host strain is a *Trichoderma* strain.

15 20. The host strain of claim 19, c h a r a c t e r i z e d in that the proteins are HFB I or HFB II or both of *Trichoderma*.

21. The host strain of any one of claims 15 to 17, c h a r a c t e r i z e d in that the host strain is a bacterial strain.

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22. The host strain of claim 21, c h a r a c t e r i z e d in that the strain is a *Bacillus spp.* strain, a *Streptomyces spp.* strain or an *E. coli* strain .

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23. A production host strain of any one of claims 15 to 22, c h a r a c t e r i z e d in that the host strain is

- genetically modified not to produce an essential amount of at least one of the proteins, polypeptides or peptides associated with foam formation during cultivation of the non-modified production host strain ; and is
- modified to be capable of producing a product of interest.

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24. The production host strain of claim 23, c h a r a c t e r i z e d in that the product of interest is a protein or a metabolite or biomass.

25. The production host strain of claim 23, characterized in that the product of interest is a recombinant product.

26. The production host strain of claim 25, characterized in that the host strain is
5 genetically modified to be capable of producing a fusion molecule comprising a molecule of interest fused to a hydrophobin.

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hfb1 genomic sequence (SEQ ID No 1)

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TTTGTATGGC TGGATCTCGA AAGGCCCTTG TCATCGCCAA GCGTGGCTAA TATCGAATGA
GGGACACCGA GTTGCATATC TCCTGATCAT TCAAACGACA AGTGTGAGGT AGGCAATCCT
CGTATCCCAT TGCTGGGCTG AAAGCTTCAC ACGTATCGCA TAAGCGTCTC CAACCACTGC
TTAGGTGACC CTTAAGGATA CTTACAGTAA GACTGTATTA AGTCAGTCAC TCTTTCACTC
GGGCTTTGAA TACGATCCTC AATACTCCCG ATAACAGTAA GAGGATGATA CAGCCTGCAG
TTGGCAAATG TAAGCGTAAT TAAACTCAGC TGAACGGCCC TTGTTGAAAG TCTCTCTCGA
TCAAAGCAAA GCTATCCACA GACAAGGGTT AAGCAGGCTC ACTCTTCCTA CGCCTTGGAT
ATGCAGCTTG GCCAGCATCG CGCATGGCCA ATGATGCACC CTTACGGCC CAACGGATCT
CCCGTTAAAC TCCCCTGTAA CTTGGCATCA CTCATCTGTG ATCCCAACAG ACTGAGTTGG
GGGCTGCGCG TGGCGGATGT CGGAGCAAAG GATCACTTCA AGAGCCCAGA TCCGGTTGGT
CCATTGCCAA TGGATCTAGA TTCGGCACCT TGATCTCGAT CACTGAGACA TGGTGAGTTG
CCCGGACGCA CCACAACCTC CCCTGTGTCA TTGAGTCCCC ATATGCGTCT TCTCAGCGTG
CAACTCTGAG ACGGATTAGT CCTCACGATG AAATTAACCT CCAGCTTAAG TTCGTAGCCT
TGAATGAGTG AAGAAATTTT AAAAACAAAC TGAGTAGAGG TCTTGAGCAG CTGGGGTGGT
ACGCCCCCTC TCGACTCTTG GGACATCGTA CGGCAGAGAA TCAACGGATT CACACCTTTG
GGTCGAGATG AGCTGATCTC GACAGATACG TGCTTCACCA CAGCTGCAGC TACCTTTGCC
CAACCATTGC GTTCCAGGAT CTTGATCTAC ATCACCAGCAG CACCCGAGCC AGGACGGAGA
GAACAATCCG GCCACAGAGC AGCACCGCCT TCCAACCTCTG CTCCTGGCAA CGTCACACAA
CCTGATATTA GATATCCACC TGGGTGATTG CCATTGCAGA GAGGTGGCAG TTGGTGATAC
CGACTGGCCA TGCAAGACGC GGCCGGGCTA GCTGAAATGT CCCCAGAGAG ACAATTGGGA
CGCTCTATGA CGGCGTGGAG ACGACGGGAA AGGACTCAGC CGTCATGTTG TGTTGCCAAT
TTGAGATTGT TGACCGGGAA AGGGGGGACG AAGAGGATGG CTGGGTGAGG TGGTATTGGG
AGGATGCATC ATTCGACTCA GTGAGCGATG TAGAGCTCCA AGAATATAAA TATCCCTTCT
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CCTCCAGTCA ACTTCCCTTA CCAGTACATC TGAATCAACA TCCATTCTTT GAAATCTCAC
CACAACCACC ATCTTCTTCA AAATGAAGTT CTTCGCCATC GCGCTCTCTT TTGCCGCCCG
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CAAAGTCCGT AAGTTGAGCC ATAACATAAG AATCCTCTTG ACGGAAATAT GCCTTCTCAC
TCCTTTACCC CTGAACAGCC TCCCAGAACG TTTACGACGG CACCGACTTC CGCAACGTCT
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CTTCTGTGCC AGACCGCCGT CCGGTGCTTGA GATGCCCCGCC CGGGGTCAAG GTGTGCCCGT
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TGTTTGGGCA GGTTTGGGCA AGTTGTGTAG ATATTCCATT CGTACGCCAT TCTTATTCTC
CAATATTTCA GTACACTTTT CTTCATAAAT CAAAAAGACT GCTATTCTCT TTGTGACATG
CCGGAAGGGA ACAATTGCTC TTGGTCTCTG TTATTTGCAA GTAGGAGTGG GAGATTGCGC
TTAGAGAAAG TAGAGAAGCT GTGCTTGACC GTGGTGTGAC TCGACGAGGA TGGACTGAGA
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TATGACTTGA TGCAATGGTG AAGATGAATG ACAGTGTAAG AGGAAAAGGA AATGTCCGCC
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CATGACATAT TTGTGGGAAC AACTTCAAAC AGCGAGCCAA GACCTCAATA TGCACATCCA
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CAAATCATCA GCTCTCATT TCGCCTAAAC CACAGATTGT TTGCCGTCCC CCAACTCCAA
AACGTTACTA CAAAAGACAT GGGCGAATGC AAAGACCTGA AAGCAAACCC TTTTGTGCGC
TCAATCCCT CTTTGTCTCT CGGAATGATG ATCCTTCACC AAGTAAAAGA AAAAGAAGAT
TGAGATAATA CATGAAAAGC ACAACGAAA CGAAAGAACC AGGAAAAGAA TAAATCTATC
ACGCACCTTG TCCCACACT AAAAGCAACA GGGGGGTAA AATGAAAT

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Fig. 1

hfb2 genomic sequence (SEQ ID No 2)

HindIII

CTCGAGCAGC	TGAAGCTTGC	ATGCCTGCAT	CCTTTGTGAG	CGACTGCATC	CATTTTGCAC
ACACTGCCGT	CGACGTCTCT	CTTCCGACCT	TGGCCAGCTG	GACAAGCAAC	ACACCAATGA
CGCTTTGTAT	TATTAGAGTA	TATGCAAGTC	TCAGGACTAT	CGACTCAACT	CTACCCACCG
AGGACGATCG	CGGCACGATA	CGCCCTCGTT	CTCATTGGCC	CAAGCAGACC	AAC TGCCCCCT
GGAGCAAGAT	TCAGCCCAAG	GGAGATGGAC	GGCAGGGCAC	GCCAGGCCCC	CACCACCAAG
CCACTCCCTT	TGGCCAAATC	AGCTTGCATG	TCAAGAGACA	TCGAGCTGTG	CCTTGAAATT
ACTAACAACC	AGGGATGGGA	AACGAAGCCT	GCTTTTGGAA	AGACAACAAT	GAGAGAGAGA
GAGAGAGGGA	GAGAGACAAT	GAGTGCCACA	AACCTGGTAG	TGCTCCGCCA	ATGCGTCTGA
AATGTCACAT	CCGAGTCTTG	GGGCCTCTGT	GAGAATGTCC	AGAGTAATAC	GTGTTTTGCG
AATAGTCCTC	TTTCTTGAGG	ACTGGATACC	TACGATACCC	TTTTTGAGTT	GATGCGGTGC
TTTCGAAGTA	TTATCTGGAG	GATAGAAGAC	GTCTAGGTAA	CTACACAAAA	GGCCTATACT
TTGGGGAGTA	GCCCAACGAA	AGGTAACCTC	TACGGCCTCT	TAGAGCCGTC	ATAGATCCTA
CAGCCTCTTG	GAGCCGTCAT	AGATCACATC	TGTGTAGACC	GACATTCTAT	GAATAATCAT
CTCATCATGG	CCACATACTA	CTACATACGT	GTCTCTGCCT	ACCTGACATG	TAGCAGTGGC
CAAGACACCA	AGGCCCCAGC	ATCAAGCCTC	CCTACCTATC	CCTTCCATTG	TACAGCGGCA
GAGAGATTGC	GATGAGCCCT	CTCCCTACCT	ACAGACGGCT	GACAATGTCC	GTATACCACC
AGCCAACGTC	ATGAAAACAA	GGACATGAGG	AACAGCCTGC	GAGAGCTGGA	AGATGAAGAG
GGCCAGAAAA	AAAAGTATAA	AGAAGACCTC	GATTCCCGCC	ATCCAACAAT	CTTTTCCATC
CTCATCAGCA	CACTCATCTA	CAACCATCAC	CACATTCACT	CAACTCCTCT	TTCTCAACTC
TCCAAACACA	AACATTCTTT	GTTGAATACC	AACCATCACC	ACCTTTCAAG	<u>ATGCAGTTCT</u>
<u>TCGCCGTGCG</u>	<u>CCTCTTCGCC</u>	<u>ACCAGCGCCC</u>	<u>TGGCTGCTGT</u>	<u>CTGCCCTACC</u>	<u>GGCCTCTTCT</u>
<u>CCAACCTCT</u>	<u>GTGCTGTGCC</u>	<u>ACCAACGTCC</u>	<u>TCGACCTCAT</u>	<u>TGGCGTTGAC</u>	<u>TGCAAGACCC</u>
<u>GTATGTTGAA</u>	<u>TTCCAATCTC</u>	<u>TGGGCATCCT</u>	<u>GACATTGGAC</u>	<u>GATACAGTTG</u>	<u>ACTTACACGA</u>
<u>TGCTTTACAG</u>	<u>CTACCATCGC</u>	<u>CGTCGACACT</u>	<u>GGCGCCATCT</u>	<u>TCCAGGCTCA</u>	<u>CTGTGCCAGC</u>
<u>AAGGGCTCCA</u>	<u>AGCCTCTTG</u>	<u>CTGCGTTGCT</u>	<u>CCCGTGGTAA</u>	<u>GTAGTGCTCG</u>	<u>CAATGGCAAA</u>
<u>GAAGTAAAAA</u>	<u>GACATTTGGG</u>	<u>CCTGGGATCG</u>	<u>CTAACTCTTG</u>	<u>ATATCAAGGC</u>	<u>CGACCAGGCT</u>
<u>CTCCTGTGCC</u>	<u>AGAAGGCCAT</u>	<u>CGGCACCTTC</u>	<u>TAAAGCAATG</u>	<u>GCTTGCTTTA</u>	<u>CTGCCGGCAG</u>
TCTTTGAGAA	CTCTGGGCTC	ACAAAAGACG	ACTTGCATGT	ATCATGGGGG	CTCGCAAAATG
GGAGGATTG	GAGGGGATTG	AGGCTGGGTT	TGGCCTATTA	GAGGATTGCA	TAATGGAAGA
TTTGCGAGCA	GGACATAGAC	GTATCTAGAG	TTCTAGTCAA	TACATTATTG	AAAAGTTGGA
GTATACCTAT	CGCTGGCACT	GGTATCTTGA	AGATATCTTC	TCTTCTTGTA	AGGTTATGTA
TGGCAATCAG	TCGAAATCTA	TTTGAAGACA	GAGCTCAAGC	TTCAAACATT	CACCTGNGAA
TTGACCATT	TGTTTCGATG	GTTCGAGTTG	GTGGGTGTCA	CTTCTGCAAT	CATGTACGAG
CACAAGTATA	GCAGTATTCC	ATCTGATCTG	CATCTGGGTA	AATGTCGCCA	CTCTACCTAG
GTACCCAATA	AATACCGAAT	TGGTCAGCTC	TCGGGTGACA	AACCGGCCCC	CTTTTCGACC
GTGCTCTGTC	CAATTCTAGG	CTTGTCAATG	GTTCTTGACT	GTGATAAACC	TTGGAGCTAN
CATAACTTAC	CTTACAATAA	ATCCAACCTG	CGGCACTTGC	TTCCCTTCAC	CCAACCACTC
GCAAACATCA	CGCAACCTGT	CTCGATCCCC	TGTCCGAAAT	CTGCTTGCCA	ACGTATCATC
ACAAATCATA	CACACAGACA	AAAAGGAGCC	AAAGCAGCAA	TGGCAAGACA	CCGAGGCCGG
CAGCGCGCCC	GTCGCCGTTT	TTAAAAAGCG	AAGCGCAAAG	GGCAAAGCCA	ACCTGCGCAA
ACGAACAACG	AAGCCTTCCC	CCCGCCGCGA	GCGACAGCGA	CAGCGACAGC	GACTTTTCCT
CGTCGGAAGA	CGAAGCCGGG	CACAGAGTCA	AGAGGCGCAA	GAGGACGGCC	GTGCTCACCG
CCGCCGCGGA	GGGGCGCCCG	GCCCAGCAAC	CGGGACGACG	GCGGCGGCGC	AACAGCCGCC
TTACCGGCCA	ACAGAAGCGT	CCCGATTGCT	GACAGCAACG	ACGCGACCAA	GCACAGCAAC
TGGTACGACG	AGGACGCAAA	GGACGCGCTC	TCGGCAAAGA	ACCTCCTCGG	ATCTTCGAGA
GCGTCCAAGG	ACGCGCAGCC	AGACGGCACG	TACAAGGGCC	TGGCGAACCA	GACGTCCTTT
ATACAAAAGA	ATCCGGATGC	GCCCCGGAAG	ACAGTTGGGC	CCGTCAAGGC	GCCTACCAAC
ATCCGCACCG	TCACCATTAC	AGATTATGCC	CCGGACACGT	GTAAAGAGTG	AGTTTGCATC
AATAGCCAGA	ATCCCCCCCC	CCGATACCGT	ACATTGAGCA	TATGCTGACT	CGTCATAATC
TTTCTAGTTA	TCGCATAACC	GGCTATATAA	GTACTCCCCT	TTTCCATGAT	TATTCCAGTC
GCGTACTGAC	ATTTCTAGGA	GCCTTTACTG	TGGTTTTGGC	GACAATTGCA	AGTATCTTCA
CGCGAGAGAA	GACCTCAAGG	CAGGCTGGCA	GCTGGATCAA	GAGTGGGAAA	AGGTCACCAA
GGGCAAGAAG	AACCTGGGGG	GAACGGTAGT	GGCCAGCGCG	AACCGGAACA	AGGCCAAGGT

Fig. 2A

GGACGAGGGC	GACGACGACG	ACGACGAAGA	GGCGATGCTC	GAGAACATTC	CGTTTGCCTG
CATCATCTGC	AGGGAATCGT	ACAAGGAGCC	GATTGTGACG	AGGTGCGGGC	ACTACTTTTG
CCTGCCGTGC	GCTCTGCAGC	GGTACAAGAA	GGATCCGACG	TGTGCGGCGT	GTGGCTCGGG
CACGAATGGC	GTGTTTAATT	CGGCGACGAG	GTTGAAGAAG	CTGCTGGAGA	AGAAGAGGGA
GAGGGCGGCC	AGGAGGAGAC	AGGAGGCGAT	AGAGAGGGGC	GAGGAAGTCA	GTGATGAAGA
GGAGGAGGAG	GAGGAGGACT	GATGATGATG	GGGCNAGATG	ACGATGCAGG	TCGACTCTAG
AGATCCCCCG	TACCGAGCTC	GAATTCATCG	<u>ATGATATCAG</u>	ATCCC	

EcoRV

Fig. 2B

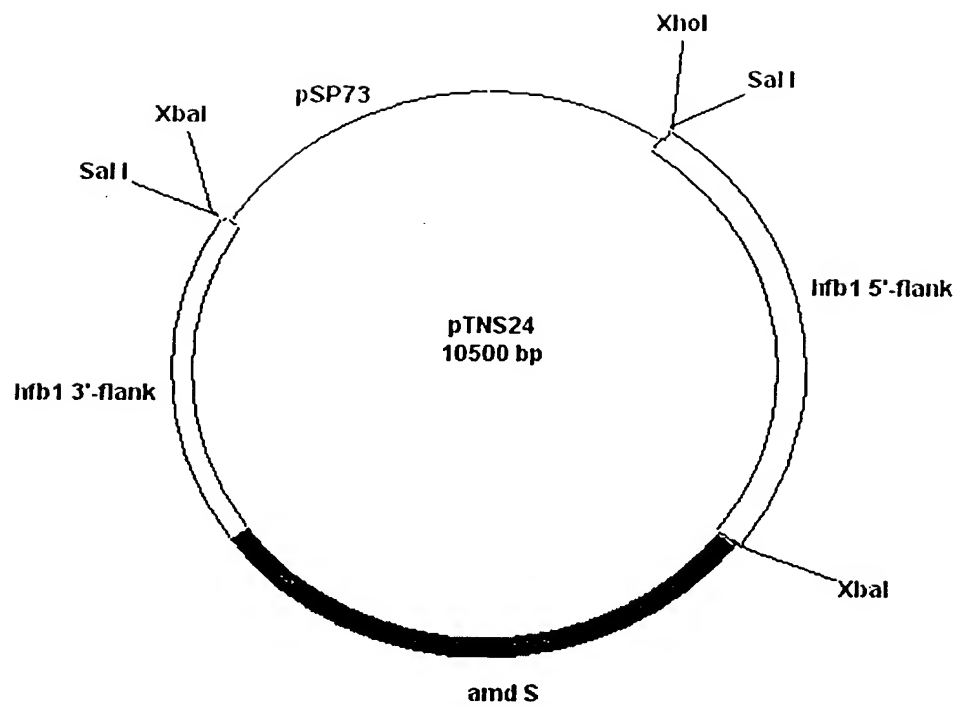


Fig. 3

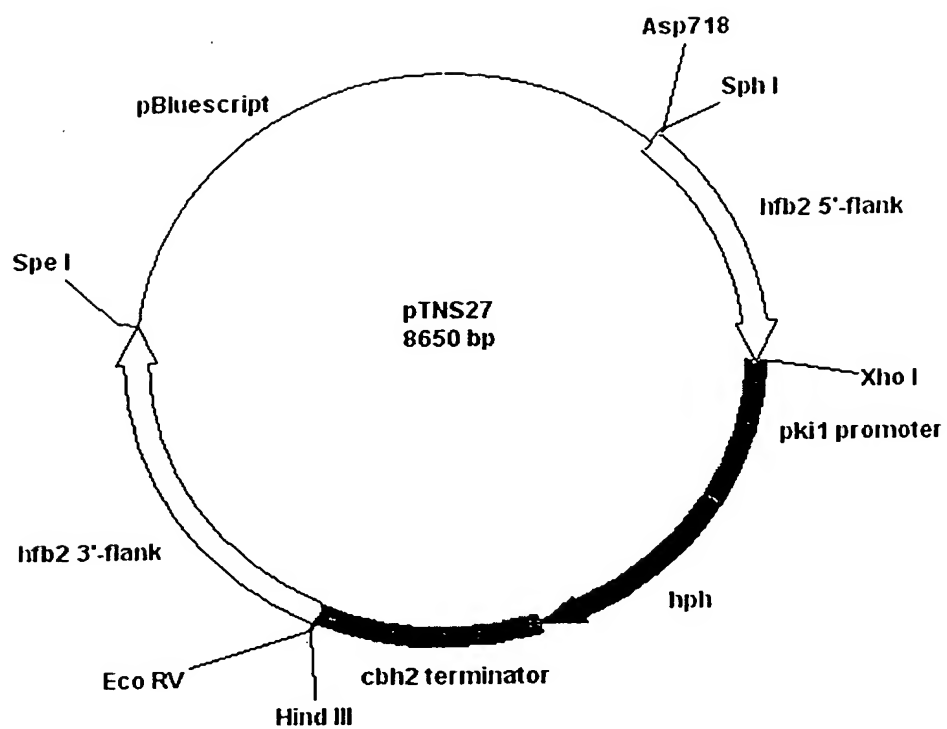


Fig. 4

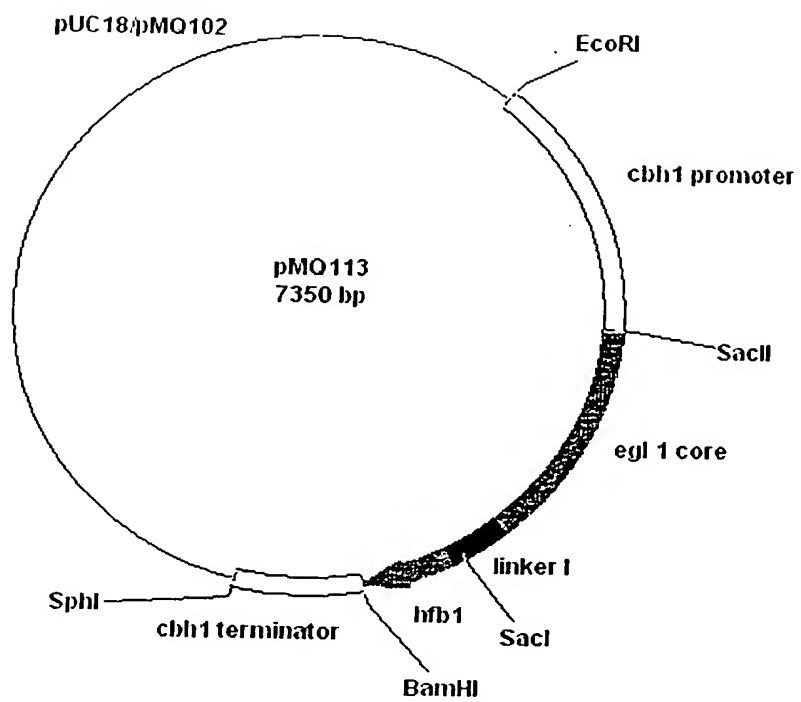


Fig. 5

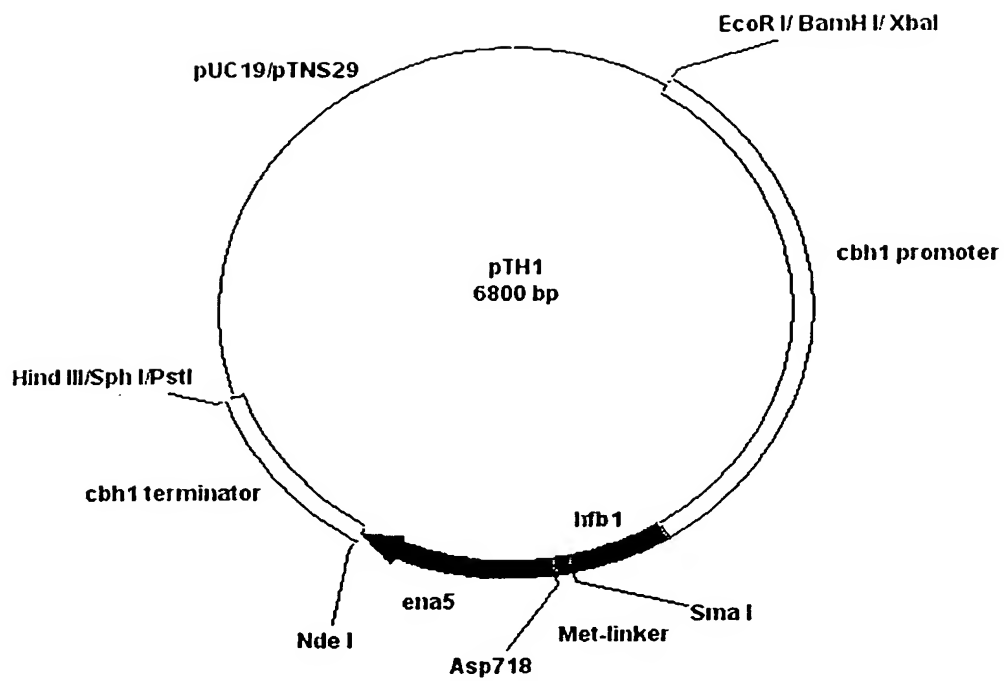


Fig. 6

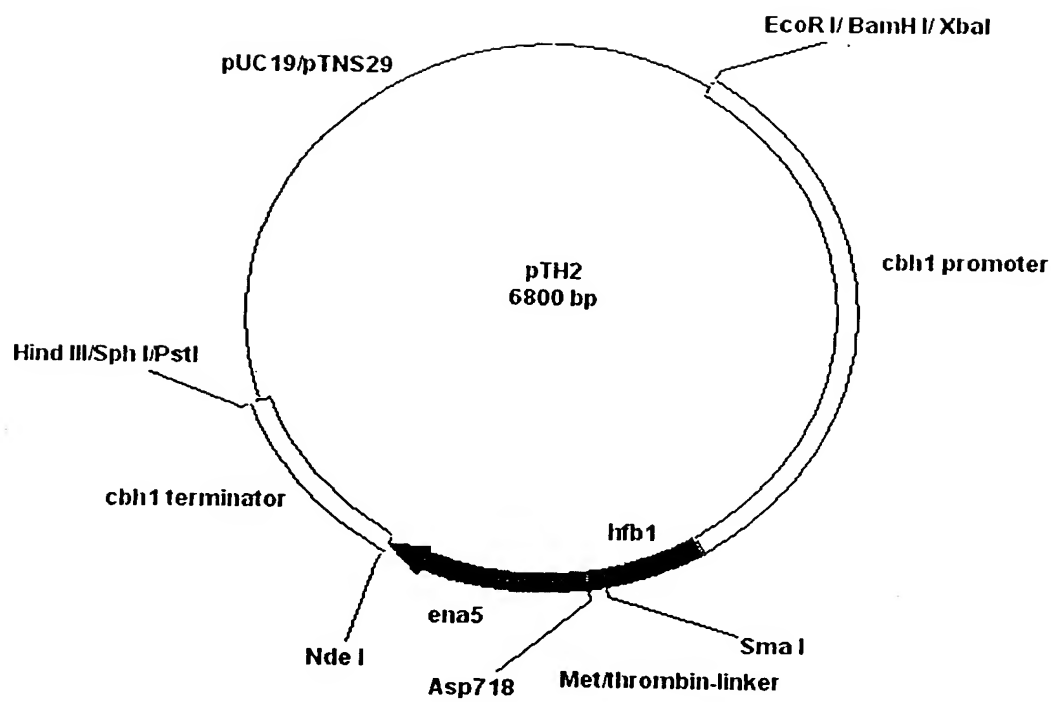


Fig. 7

Strain Rut C30

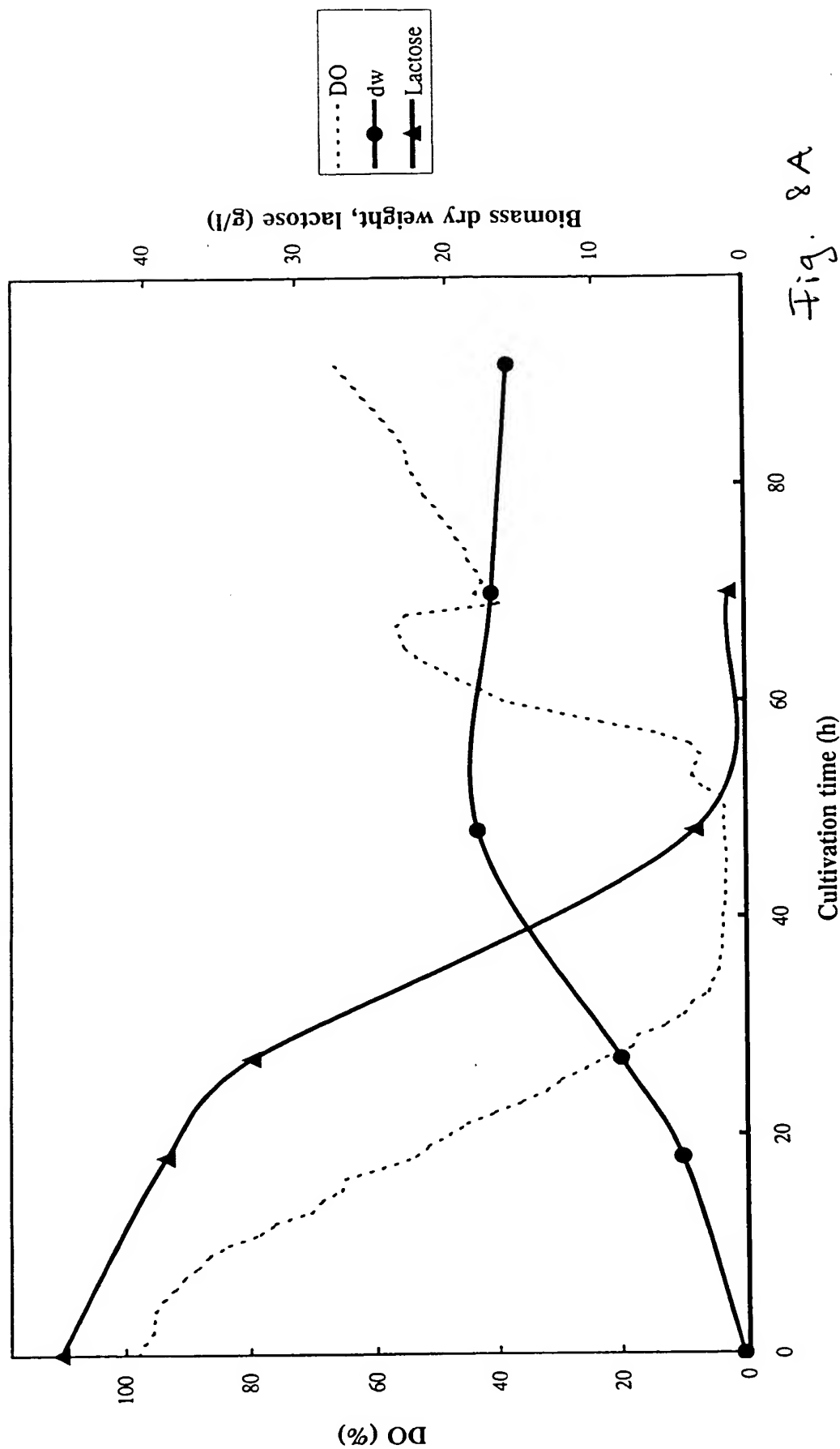


Fig. 8A

Strain VTT D-99676

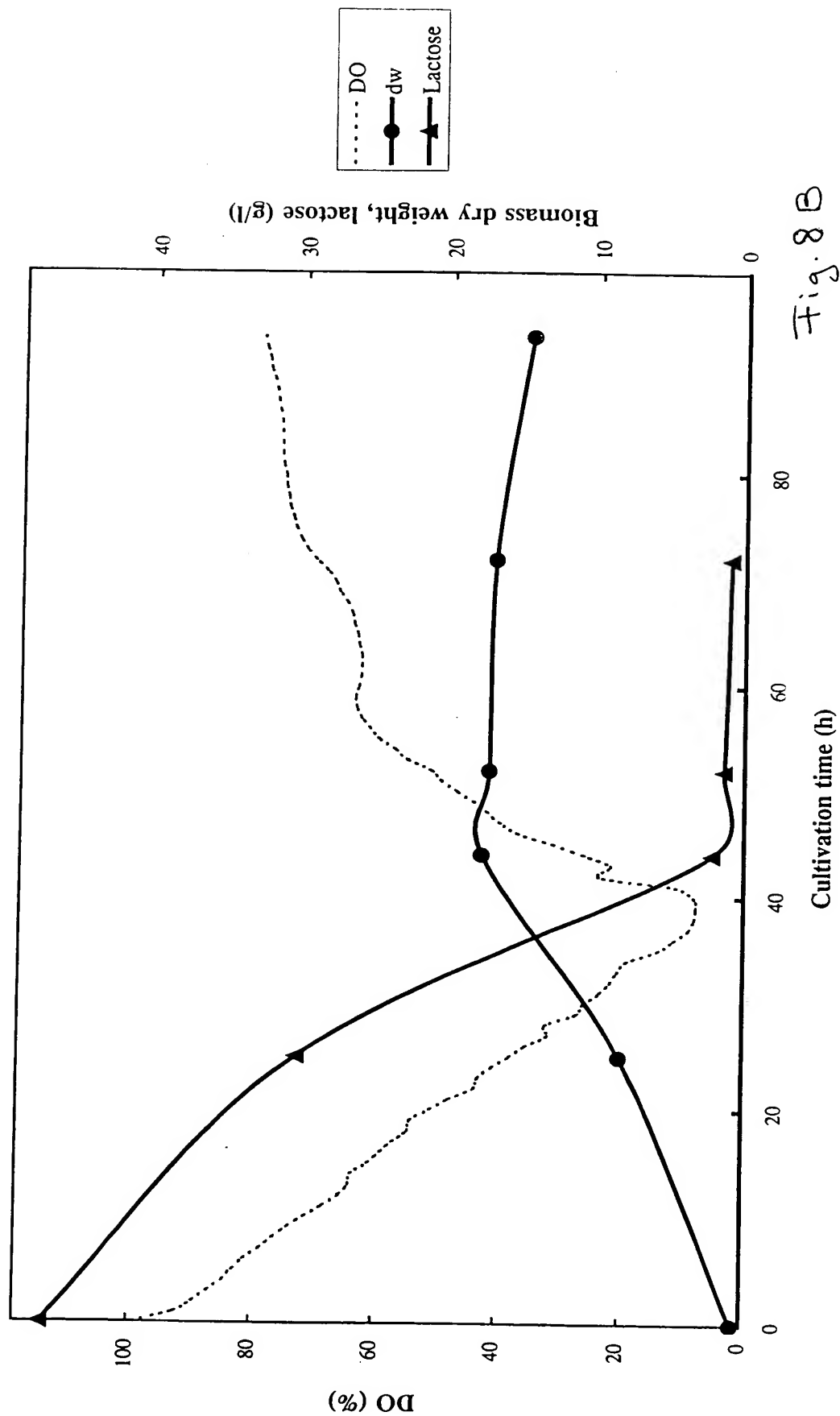


Fig. 8B

Strain Rut C30

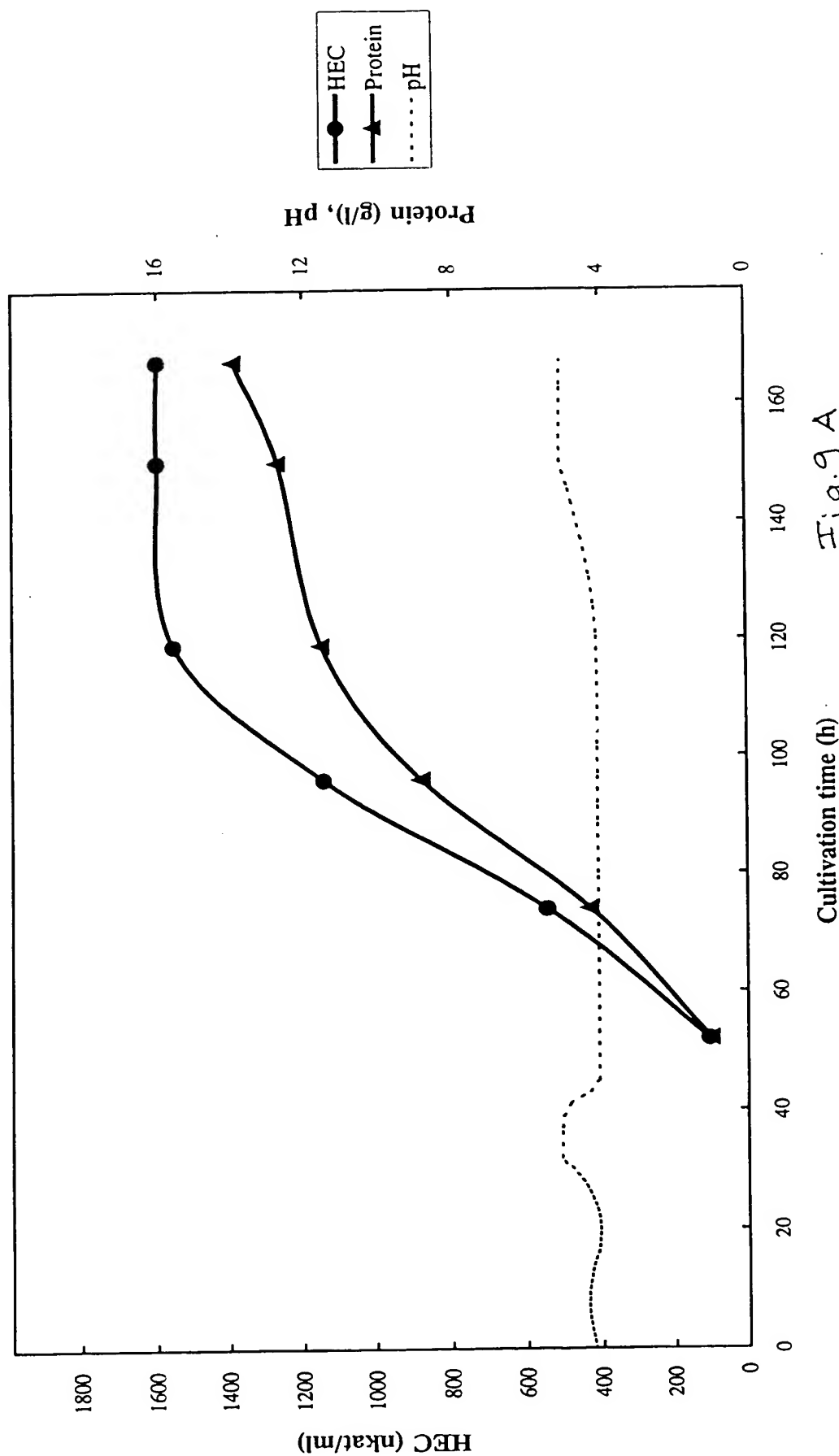


Fig. 9 A

Strain VTT D-99676

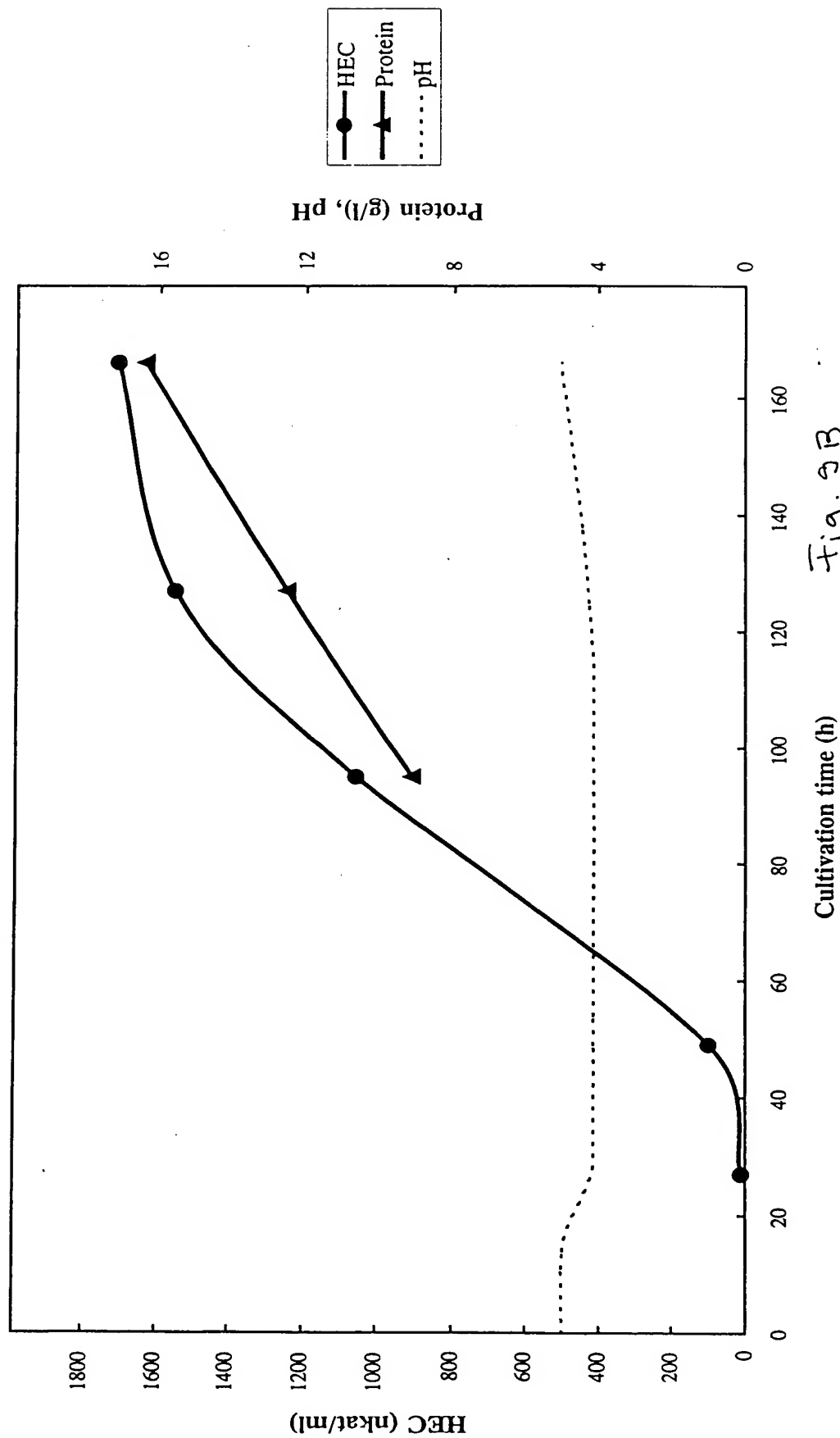


Fig. 9B